Endophytic Fungi Associated with Pioneer Plants Growing on the Athabasca Oil Sands

By

Xiaohui Bao

Department of Biology

University of Saskatchewan

In partial fulfillment for the Degree of Master of Science

PERMISSION TO USE

ABSTRACT

Fungal endophytes live inside plants without causing apparent symptoms of infection. All plant species surveyed thus far, including liverworts, mosses, seedless vascular plants, conifers, and angiosperms, harbor one or more endophytic fungi. Fungal endophytes can be divided into four groups including class 1, class 2, class 3 and class 4 endophytic fungi according to host range, colonization pattern, transmission, and ecological function. Class 2 fungal endophytes benefit their host by increasing environmental stress tolerance (i.e. water, temperature, salt) in a habitat-specific manner. In my study, class 2 fungal endophytes were studied from weedy plants growing in an environmentally stressed area: mine tailings from the Athabasca oil sands. This area is a vast hydrocarbon reserve in western Canada that supplies 10% of Canadian oil needs. Hydrocarbons are extracted from tar sands with hot water, alkali, and solvents. The tailing sands can later be remediated (by adding organic material and fertilizer) to establish new plant communities. Prior to remediation, tailing sands have extremely low content of organic carbon and available minerals, and are hydrophobic compared to unimpacted and remediated soils. Nevertheless, *Taraxacum* (dandelion) and *Sonchus* (sow-thistle) can colonize extracted tailing sands even prior to remediation. Preliminary results show that pioneer plants have similar fungal abundance as plants of unextracted treatments. Fungal endophytes were isolated from surface sterilized *Taraxacum* and *Sonchus* that had been growing upon unimpacted, remediated and extracted soil. Fungi isolated in this way included *Alternaria*, *Tricoderma*, *Fusarium* and an unidentified Perithecial Ascomycote. These endophytic fungi were used to inoculate tomato plants in a greenhouse trial to determine whether they confer stress tolerance to host plants, especially for drought and low mineral nutrition. Before exposing the tomato plants to environmental stresses, the specific endophytic fungal strains applied were successfully

recovered from tomato plants originally inoculated with the same endophytic fungi. Although the other endophytic fungi turned out to be harmful to the tomato plants in the test, a *Trichoderma* spp. strain isolated from samples of extracted treatment appears to confer tolerance of tailing sands to the tomato plants. This *Trichoderma* spp. strain which we can call TSTh20-1was molecularly identified as *Trichoderma harzianum*. Despite an identification to species, all strains of T. *harzianum* are not necessarily identical regarding strain-specific attributes. Using similar techniques described here, it is possible to isolate and potentially use beneficial class 2 endophytic fungal strains for the remediation process in the Athabasca oil sands or to assist plant growth in other high stress environments.

ACKNOWLEDGMENTS

I am deeply indebted to my supervisor, Dr. Susan Kaminskyj, for her constant support and encouragement as well as excellent advice through out my study. Without her help, this work would not be possible. I would like to thank my advisory committee members Drs. Jill Johnstone and Som Niyogi for their suggestions, criticism, and patience throughout my graduate program. I would also like to thank Drs. Russell Rodriguez and Regina Redman, technical direction from them was really appreciated. This project could not have been done without the soil samples collected from the Athabasca oil sands by Blair Hersikorn and Judit Smits, and plant samples collected by Dr. Ali Quoreshi. A special thanks to Dr. Jim Basinger for his helpful advice and support.

Molecular experiment guidance from Drs. Yangdou Wei and Guosheng Liu was invaluable. Whenever I had trouble with PCR, they were always there to help. Special thanks to Dr. de Freitas in the Department of Soil Science, he helped me with my entire soil assay work and answered my questions with patience. I would like to thank the Department of Biology, University of Saskatchewan for providing the financial support and the nice research atmosphere. An acknowledgment also goes to graduate students in the Department of Biology, especially Lilia Li, Michelle Hubbard, Amira El-Ganiny, and Sanjaya Ekanayake.

I would like to deeply thank my parents for their support, both financially and mentally. My Canadian family also deserves a deep thanks for all the support. Also, a heavy thank you to my husband, Glyn McMillan, who is ever patient and assists me whenever possible.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABA	Abscisic Acid
act	Actin
AFLP	Amplified fragment length polymorphism
AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
B	Boron
BLAST	Basic local alignment search tool
Ca	Calcium
$CaCl_2$	Calcium chloride
cal	Calmodulin
CLSM	Confocal laser scanning microscopy
CO_2	Carbon dioxide
Cu	Copper
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double distilled water
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DSE	Dark septate endophyte
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid

EF-1α	Elongation factor gene
Fe	Iron
FE	Fine endophyte
FTIR	Fourier Transform Infrared
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ITS	Internal transcribed spacer
K	Potassium
K_2O	Potash
KCl	Potassium chloride
KOH	Potassium hydroxide
LF	Lactofushsin
Mg	Magnesium
$MgCl_2$	Magnesium chloride
Mn	Manganese
Mo	Molybdenum
MQM	Multiple quantitation method
N	Nitrogen
Na	Sodium
$NaHCO_3$	Sodium bicarbonate
NA	Naphthenic acid
$(NH_4)_2SO_4$	Ammonium sulfate
OD	Optical density
P	Phosphorus

PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
P_2O_5	Phosphoric acid
ppm	Parts per million
PVAG	Polyvinyl alcohol
RAPD	Random amplification of polymorphic DNA
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal RNA
S	Sulfur
SE	Septate endophyte
spp	Species
SSU	Small subunit
Tris-HCl	Tris (hydroxymethyl) aminomethane-hydrochloric acid
T-22	*Trichoderma harzianum* Rifai 1295-22
UV	Ultra violet
Zn	Zinc

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

1.1. Symbiotic relationship between plants and fungi

Symbiosis is a mutually beneficial interaction between two organisms. Most plants including liverworts, mosses, seedfree vascular plants, conifers, and angiosperms are symbiotic with fungi (Rodriguez et al., 2004; Arnold and Lutzoni, 2007) and symbiotic fungi play important roles in the structure, function, and health of plant communities (Petrini, 1986; Bacon and Hill, 1996; Rodriguez and Redman, 1997; Clay and Holah, 1999; Read, 1999;). There are two major groups of fungal symbionts associated with plants: mycorrhizal fungi that reside not only in roots but also extend out into the rhizosphere; and fungal endophytes that reside entirely within plant tissues and may be associated with roots, stems and/or leaves. Both types of interaction are extremely ancient, having been documented in Early Devonian fossils from the Rhynie chert (Krings *et al*., 2007).

Arbuscular mycorrhizal fungi (AMF) have aseptate hyphae, produce characteristic intracellular structures (arbuscule or coil) within roots, are transmitted by means of asexual spores in the soil, and are obligate biotrophs. The latter means that their growth requires another living organism. For symbiosis between AMF and terrestrial plants, AMF receive shelter and photosynthetic carbohydrates from the host plants while the host plants receive mineral nutrients from AMF (Simon, 1996). AMF are also known for enhancing host plant tolerance to water deficit (Koske and Gemma, 1997; Augé, 2001), high heavy metal concentration (Schutzendubel and Polle, 2002; Goehre and Paszkowski, 2006), some kinds of soil-borne pathogens and toxicants in the soil (Norman *et al*., 1996; Leung *et al*., 2007).

Septate endophytes (SE) have septate hyphae and colonization of host plants, via sporulation (generation of spores), occurs by vertically and horizontally transmitting spores. Vertical transmission of SE spores is associated with host plant senescence and spores are transmitted to the next plant generation via the seed coat, and rhizomes (Rodriguez *et al*., 2008a). SE spores can also be horizontally transmitted to nearby plants by means of rain, wind, or direct contact (Rodriguez *et al*., 2008a). Similar to AMF, SE fungi gain shelter and nutrients from host plants. Various functional classes of SE are known to provide benefits to the host plant including reduced animal herbivory and assist host plants to adapt to high-stress environments (Wilson, 1995; Rodriguez *et al*., 2004; Lehtonen *et al*., 2005) as well as improve their host's competitive ability (Clay *et al*., 1993).

Relative to the amount of research about the symbiotic relationship between AMF and terrestrial plants, much less work has focused on how the endophyte fungal symbiont promotes the growth of host plants. Porter *et al* (1979) described early research by Robbins and Bacon (1976) where endophytic fungi were described in grasses (such as tall fescue, *Festuca arundinacea*) growing on a pasture, however, the function of endophytic fungi (such as *Neotyphodium* spp.) was not yet clear. It is now known that cool season grasses infected by the fungal endophyte *Neotyphodium* spp. are more vigorous, more toxic to herbivores, and more resistant to drought compared to uninfected cold season grasses (Rice *et al*., 1990; Clay and Holah, 1999; Malinowski and Belesky, 2000).

Four classes of endophytic fungi have now been described (Rodriguez *et al*., 2008a), each of which are different in their host range, colonization pattern, transmission, and ecological function (Rodriguez and Redman, 2008). As a typical example of class 1 endophytes, Clay and Schardl (2002) suggested that the grass fungal endophytes which are found in cold-seasoned grass may constitute a monophyletic clade with the fungal family Clavicipitaceae, whereas the other 3 classes were known as non-Clavicipitaceous. Both mycorrhizae and Clavicipitaceae (class 1) endophytes have been well studied, while less is known about non-Clavicipitaceae (class 2-4) endophytes, which may represent a relatively large group of fungal symbionts, particularly class 2 (Rodriguez and Redman, 2004; Rodriguez et al., 2008a).

The Rodriguez group's research has focused on class 2 endophytic fungi that have a wide range of host plants, implying a vast potential for application. Although class 2 endophyte fungi have not been as extensively studied, their lab has already provided evidence to show that class 2 endophyte fungi confer the tolerance of host plants to extreme environments including high temperature, drought and high salt content (Redman *et al*., 2002; Rodriguez *et al*., 2004; Rodriguez and Redman, 2007). A fungus strain in *Curvularia protuberata* was shown to assist host plants to adapt to geothermal soil (Redman *et al*., 2002), a *Colletotrichum magna* strain conferred the tolerance of host plants to drought (Redman *et al*., 2001) and *Fusarium culmorum* has been shown to confer salt tolerance to the host plant (Rodriguez *et al*., 2008b). Fungal endophytes not only promote stress-tolerance, but also increase nutrient acquisition and growth rates of their host (Rodriguez *et al*., 2004).

1.2. Arbuscular mycorrhizal fungi (AMF)

1.2.1. Definition

Arbuscular mycorrhizal fungi (AMF) are a group of common soil-borne fungi that are associated with the roots of over 80 % of all terrestrial plants. As the most widespread type of mycorrhiza, this symbiosis is highly significant on a global scale. Although AMF are obligate biotrophs, the arbuscular mycorrhiza association is a symbiosis that benefits both partners. The fungal mycelium absorbs mineral nutrients from soil, including N, P, K, Ca, S, Fe, Mn, Cu, and Zn (Garg *et al*., 2006), which are necessary for the growth of host plants (Smith and Read, 1997;

Vierheilig *et al*., 1998; Akiyama *et al*., 2005). A commonly accepted opinion is that increased phosphate uptake in plants colonized by AMF depends mainly on the external hyphae that grow into the soil outside of the root's P depletion zone which develops around the root. Growing roots generally deplete the rhizosphere of soluble phosphate more quickly than replenishment can take place (Kraus *et al*., 1987; Tawaraya *et al*., 2006).

1.2.2. Classification

AMF were previously placed in the class Glomeromycetes within the division Zygomycota based on the morphological similarities, for example, the interpretation of AMF spores as azygospores, which are zygospores with only one gametangium (Walker, 1983; Morton and Benny, 1990). Currently, AMF are placed into their own phylum, the Glomeromycota, based on molecular evidence of the small subunit (SSU) ribosomal RNA (rRNA) gene (Figure 1.2.2) (Schussler *et al*., 2001). AMF were identified to genus based on methods which focused on the morphology of the asexual spore. Spore wall structure (i.e. color, ornamentation pattern, thickness, histochemical reactivity) and spore development offer most of the information to identify AMF in this method (Bentivenga and Morton, 1994; Franke and Morton, 1994).

Obtaining AMF spores from environmental samples can be inconsistent due to variable spore number, parasitization of spores, spore viability, environmental alteration of spores such as discoloration and season-dependent spore density, which can lead to inaccurate identification (Bever *et al*., 2001). Since AMF are obligate biotrophs, they cannot be cultured in the absence of host plant roots (Bago and Bacard, 2002) so highly limited sources of obtaining AMF spores severely hampers large scale experiments using AMF. In contrast to the limitations of classical morphological identification techniques, using molecular methods in AMF identification is extremely valuable. Typically, the nucleic information derived from the AMF is from ribosomal DNA (rDNA) (Redecker, 2000a). The internal transcribed spacer (ITS) region comprises highly conserved functional sequences and more variable spacers that are not under selective pressure. ITS regions are used for the identification or phylogenetic analyses of a wide range of fungi and fungus-like organisms (Redecker *et al*., 1997; Bever *et al*., 2001). Redecker (2000a) designed group-specific primers to amplify the ITS and 18 S rRNA gene fragments for identifying major subgroups of AMF. These group-specific primers have been widely used and proved to be effective for identifying AMF collected in field (Redecker *et al*., 2003; Wubet *et al*., 2003; Hijri *et al*., 2006; Shepherd *et al*., 2007). Several new genera and families have been erected recently (Walker and Schussler, 2004; Walker *et al*., 2007) and AMF species are placed under seven genera: *Acaulospora, Entrophospora, Archaeospora, Glomus, Paraglomus, Gigaspora* and *Scutellospora* (Khade, 2008).

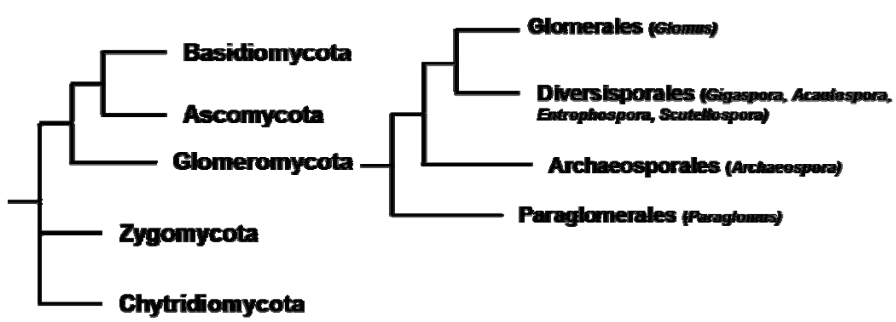

Figure 1.2.2. Phylogenetic tree of fungus (left) showing division of Glomeromycota (AMF and relatives). Based on work from Schussler *et al*., (2001) and Khade (2008).

1.2.3. Ecology and Evolution

Early ancestors of modern AMF have been found in Rhynie chert fossils formed in Early Devonian (410 million years ago) (Strullu-Derrien and Strullu, 2007), which show that the rhizoids of *Nothia* have fungal colonization and the hyphae of some mutualistic colonists were encased by plant cell walls (Berbee and Taylor, 2007). Although not showing an association with plant tissue, the earliest fossil evidence of AMF, represented as entangled, occasionally branching, nonseptate hyphae and spores, were found in rocks over 460-455 million years old (Redecker *et al*., 2000b). All of the spores in the fossils retain their subtending hyphae which have a diameter of 3-5 μm. The spore wall consists of a single layer. According to their shape, size, and hyphal connection, the spores are very similar to those of modern fungi in the genus *Glomus*. Also, the loose spore clusters that were found in the fossils are frequently formed by AMF but are unknown from algae, other fungi, or Oomycetes (Redecker *et al*., 2000b). This evidence implies that the fungi found in the fossils strongly resemble modern AMF.

Fossil evidence strongly suggests that major groups of fungi were present even before the first terrestrial vascular plants that arose in the Silurian period (444-417 million years ago) (Redecker *et al*., 2000b). In terms of time, this supports the hypothesis that AMF association may have assisted ancestral vascular plants to invade land (Pirozynski and Malloch, 1975). During the Silurian period, it is proposed that ancestors of modern plants lacked the ability of extracting essential nutrients from the soil or perhaps sufficient quantities. Since the ancestors of modern AMF did not have the ability of producing carbohydrates (Pirozynski and Malloch, 1975), the relationship was established between these two so that both of the partners could adapt to the terrestrial habitat.

1.2.4. Development of AMF symbiosis

In the modern day, the sequence of steps leading to the establishment of a functional symbiosis between AMF and host plants involves: (I) the presymbiotic phase; (II) contact and entrance of the fungus into root tissue; (III) intraradical fungal proliferation; and (IV) cell invagination and nutrient transfer (Paszkowski, 2006). In the presymbiotic stage, the fungus senses a host-derived signal (such as strigolactones) leading to intensified hyphal branching that is possibly to increase the chance of contact with a host root (Akiyama *et al*., 2005). The concentration of strigolactone in root exudates coincides with the host specificity of AMF so that the host plant can be recognized. For example, the non-mycotrophic plant *Arabidopsis thaliana* produces very low amounts of strigolactones compared to hosts of AMF (i.e. carrot and tobacco) (Westwood, 2000). After host plant recognition, AMF hyphae penetrate root hair cell or epidermal cells through exerting a hydrostatic pressure at the hyphal tip as well as by producing cell wall degrading enzymes to get through the localized cell wall (Bonfante and Perotto, 1995). For example, G*lomus mosseae* showed higher activities of cell wall degrading enzymes such as pectinase in AMF colonized roots compared to non-symbiotic roots (Garcia-Romera *et al*., 1991; Garcia-Garrido *et al*., 1992). After AMF successfully penetrate into the host roots, their aseptate hyphae grow through the cortex in both directions from the entry point. The host response to intracellular hyphal invasion such as the formation of arbuscules or hyphal coils is a forming of a host-derived membrane that is continuous with the plant cell membrane (Bonfante and Perotto, 1995).

1.2.5. Typical fungal structures in AMF symbiosis

AMF produce characteristic intracellular structures within roots that are readily distinguished with confocal laser scanning microscopy (CLSM). Arbuscules and/or hyphal coils are observed in AMF infected plant roots. Vesicles are also a typical AMF structure found in AMF infected plants.

1.2.5.1. Arbuscules and hyphal coils

The highly branched tree-like fungal structures which are formed by repeated branching of intracellular hyphae within root cortical cells were named ‘arbuscules’ by Gallaud (1905). Hyphal loops inside of plant cell are called hyphal coils which are frequently found growing in the outer cortex (Cavagnaro *et al*., 2001). The formation of an arbuscule starts from a trunk hypha (5-10 μm in diameter) proliferating fine branch hyphae (<1 μm diameter). The formation of arbuscules is typically accompanied with plant vacuole fragmentation and the movement of the nucleus to the center of the cell (Bonfante and Perotto, 1995). One of the significant functions of arbuscules and hyphal coils is to increase the contact area between the fungus and cortical cells of the root. The arbuscules and hyphal coils are considered to be the structures responsible for the exchange of carbohydrates and mineral nutrients between AMF and host plants (Smith and Smith, 1990).

1.2.5.2. Vesicles

Vesicles are not present in every AMF symbiosis and it is thought that some AMF species don’t produce them at all (Smith and Smith, 1997). Vesicles are hyphal swellings in the root cortex that contain lipids and nuclei (Smith and Smith, 1997). Vesicles have the function of storage and when they are old, they also play the role of reproduction (Miyasaka and Habte, 2001). Biermann and Linderman (1983) successfully inoculated previously uninfected roots using vesicles of some species of AMF as propagules. Some research shows vesicles start to grow soon after the arbuscules are formed and continue to develop when the arbuscules degenerate (Peterson *et al*., 2004).

1.2.6. AMF benefits

AMF benefits to host plants are related to assisting the host plants to increase the uptake of mineral nutrients, especially phosphorus (P). P is an important plant macronutrient building molecule, because it is necessary for producing nucleic acids and phospholipids, and for regulating enzymatic and metabolic reactions (Theodorou and Plaxton, 1993). Compared to plant roots, AMF are much more efficient in capturing P (Bolan, 1991). Cox and Tinker (1976) measured the quantity of P transferred per unit time then divided it by the area of the interface (measuring mosaics of electron micrographs from fixed transverse sections) to estimate the P flux at 13 $nmol/m^2/s^1$ along the hyphae in mycorrhizae. This P transfer speed is up to six times faster than the P transfer speed in the root hairs. Additionally, the extraradical hyphae grown into the surrounding soil increase the surface area of soil contact which also leads to more effective uptake of P. AMF may also improve soil phosphorus availability through solubilizing inorganic forms of phosphorus and mineralization of organic phosphorus (Hamel, 2004; Tawaraya *et al*., 2006).

1.2.7. Limitations for application of AMF

AMF are obligate biotrophs, that is, they cannot be cultured without an intimate relationship with living host plant roots and the single-spore pure culture cannot be developed. The obligate characteristic of AMF makes it extremely difficult for selecting and identifying highly effective AMF strains that have the potential of being used for either enhancing the growth of host plants or assisting environmental remediation. This obligate nature also limits the amount of spores that can be produced in lab which severely hamper the large scale application of AMF. Another limitation for the application of specific AMF association for remediation is that the beneficial effects depend on not only the combined efficacy of plants and fungi involved but also on their ecological interactions within the system (soil, water, sediment, etc.) which makes the mechanism even more complicated and more factors need to be considered in the application procedure of AMF (Kapoor *et al*., 2008).

1.3. Endophyte fungi

1.3.1. Definition

In contrast to AMF that only colonize in the host root, fungal endophytes reside within roots, stems and/or leaves and emerge during host senescence (Saikkonen *et al*., 1998). Endophyte fungi are considered as plant mutualists and live asymptomatically within the host tissues, receiving protection and nutrients, while host plants may benefit from increased resistance to herbivores, pathogens, and various abiotic stresses as well as enhanced competitive abilities (Wilson, 1995; Rodriguez and Redman, 1997; Lehtonen *et al*., 2005).

1.3.2. Endophytic fungi classification and host range

Endophytic fungi consist of a phylogentically diverse group that are members of the dikaryomycota (Carroll, 1988; Schardl *et al*., 2004; Van Bael *et al*., 2005; Girlanda *et al*., 2006; Arnold and Lutzoni, 2007). While most endophyte fungi belong to the Ascomycota, a few belong to the Basidiomycota (Rodriguez *et al*., 2008a). Fungal endophytes can also be discriminated into different functional groups similar to what has been done with mycorrhizal fungi (Brundrett, 2006). Currently, endophytes are subdivided into 4 classes including class 1, class 2, class 3 and class 4 according to host range, colonization pattern, transmission, and ecological function (Rodriguez and Redman, 2008).

Class 1 endophytes are a relatively small number of species that are limited to a few monocot hosts and class 2-4 endophytes are a large number of species with broad host ranges, including both monocots and dicots (Rodriguez and Redman, 2004). Class 2 endophytes can grow in both above and below ground tissues and form extensive tissue colonization, while class 3 endophytes are restricted to above ground tissues and form highly localized infections and class 4 endophytes are restricted to roots (Rodriguez *et al*., 2008a). Only class 2 endophytes have been shown to confer habitat-adapted stress tolerance, although most class 3 and 4 endophytes have not been assessed for conferring fitness benefits to hosts (Rodriguez *et al*., 2008a). Class 2 fungal endophytes not only have a broad host range, but have been shown to confer the adaptation of host plants to high-stressed environments and are typically of high infection frequencies in plants growing in high stress habitats (Redman *et al*., 1999a, 2001, 2002; Rodriguez and Redman, 2007). Based on these qualities, I hypothesized that class 2 endophytic fungi may assist plant growth in the Athabasca tailing sands (a high abiotic stress environment).

Abiotic stress is the negative impact of physical and chemical factors (i.e. temperature, rainfall, pH, oxygen content, etc.) on living organisms in a specific environments, which is essentially unavoidable and naturally occurring. The Athabasca tailing sands are a high-stress environment that limits plant growth due to major abiotic stress factors such as hydrocarbon residues, alkali that is widely used in the oil extraction process, and extremely low mineral nutrient content.

1.3.3. Development of class 2 endophyte symbiosis

Similar to other endophytes, class 2 endophtyic fungi colonize plants by using hyphae to penetrate into the plant tissue or through infection structures such as appressoria (Ernst *et al*., 2003). The growth of the fungi through the plant tissues in primarily intercellular with little to no injury on host cells (Rodriguez *et al*., 2008a). Sporulation occurs rapidly by means of emergence during host senescence (Weber *et al*., 2004).

1.3.4. *In vitro* culture of endophytic fungi

Unlike AMF, which cannot be cultured on an artificial medium, endophytic fungi can be isolated and easily cultured from plant tissue on artificial medium. In addition, endophyte fungi reside entirely within plant tissues or associated with roots, stems and/or leaves, so endophyte fungi can be isolated from different parts of the plant on culture medium plates.

1.3.5. Molecular identification of endophytic fungi

1.3.5.1. Target DNA sequences

The internal transcribed spacer (ITS) region of fungal rRNA genes has been identified by previous studies as suitable targets for molecular analysis of fungal identification (Bridge and Spooner, 2001; Gardes and Bruns, 1993). ITS regions are stretches of DNA that are located between the 18S and 5.8S ribosomal DNA (rDNA) coding regions (ITS1) and between the 5.8S and 28S rDNA coding regions (ITS2) (Bakker *et al*., 1992) (see figure 1.3.5.1). The ITS region has a high degree of variation between species because of the relatively low evolutionary pressure acting on these non-functional sequences. Their highly varied sequence relative to the conserved flanking rRNA genes in fungi and the high copy number of rRNA genes makes it to be easy to amplify even from small quantities of DNA (Buchan *et al*., 2002). In my project, ITS sequence data were used as the complement of morphological methods to identify the endophyte fungi strains at genus level.

To identify the *Trichoderma* strain to species level, sequence information of an approximately 0.65 kb fragment of the protein coding translation elongation factor gene (EF-1α) (Figure 1.3.5.2), 0.5 kb section calmodulin gene (cal), 0.75 kb actin gene (act), and ITS can be combined together to provide a much greater resolution of the representatives of *Trichoderma* spp. compared to what ITS sequence alone supplies (Samuels *et al*., 2006). This method makes it possible to identify the *Trichoderma* strain to species level accurately.

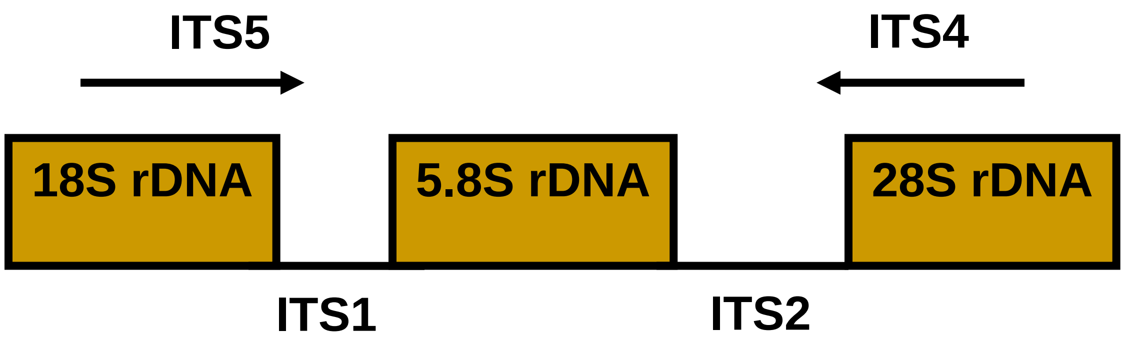

Figure 1.3.5.1. The amplification sites of universal primers (not to scale).

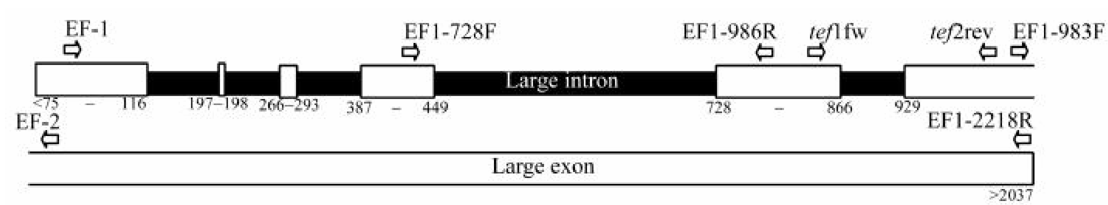

Figure 1.3.5.2. A schematic structure of EF-1α gene and location of primers used for amplification of different parts. From Druzhinina and Kubicek (2005).

1.3.5.2. Primer design

The primer pair ITS4 and ITS5/ITS1 is universal primer pair used for the amplification of the ITS1-5.8S-ITS2 target sequence (White *et al*., 1990) (Figure 1.3.4.1). O'Donnell *et al*. (1998) designed primer pair EF-1 and EF-2 to amplify translation elongation factor coding region (EF-1α) from *Fusarium spp*. Then primer pair EF1-728F and TEF1 rev was applied in amplification of EF-1α coding region in *Trichoderma spp* and showed positive results (Samuels *et al*., 2002). CAL-228F and CAL-737R primer pair were designed to amplify calmodulin gene in filamentous fungi by Carbone and Kohn in 1999, then used in identification of *Trichoderma spp* by Chaverri *et al*. (2003b). Tact1 and Tact2 primer pair were designed and successfully used for amplifying actin gene in *Trichoderma spp* by Samuels *et al*. (2006).

1.3.5.3. Polymerase chain reaction (PCR)

PCR is a technique widely used in molecular biology for its efficiency in amplifying the target sequence from small quantities of DNA and RNA templates (Van Tuinen *et al*., 1998; Schwarzott *et al*., 2001). The PCR procedure consists of four steps: 1) Denaturation 2) Annealing 3) Elongation and 4) Final elongation is performed to ensure any remaining single-stranded DNA is fully extended (Sambrook and Russell, 2001). Almost all PCR applications involve a heat-stable DNA polymerase, such as thermal *Taq* polymerase (an enzyme originally isolated from the thermophilic bacterium *Thermus aquaticus*), which assembles a new DNA strand from the nucleotides by using single-stranded DNA as a template and DNA primers as initiation.

1.4. Class 2 endophyte fungi and plants

Class 2 endophytes are different from other classes of endophyte fungi as they can form extensive colonization in roots, stems and leaves, are vertically transmitted by means of seed coats and/or rhizomes as well as horizontally transmitted by rain or air, and have a low abundance in the rhizosphere (Rodriguez *et al*., 2008a). Class 2 endophyte fungi are also unique for enhancing habitat-specific stress tolerance of host plants (Redman *et al*., 2002; Rodriguez *et* *al*., 2004; Rodriguez and Redman, 2007) and have high colonization rates (90-100 %) in plants growing in high stress habitats (Rodriguez *et al*., 2008a).

According to the relationship and mechanism between endophyte fungi and their host plants in high stress habitats, Rodriguez *et al*., (2008b) developed the theory of habitat-adapted symbiosis which suggests that endophyte fungi confer specific fitness benefits to host plants growing in harsh environments. The tailing sands are not optimal for plant growth because of low mineral nutrient content, low organic C, high pH, and residual hydrocarbon (Bois *et al*., 2006). All of these abiotic stress factors imply that a habitat-adapted symbiosis between class 2 endophytic fungi and pioneer plants can be established in Athabasca tailing sands.

1.4.1. Endophytic fungi benefits

Cool-season grass infected by class 1 endophyte fungi show better tolerance to biotic and abiotic stresses and become more competitive than non-infected grass (Marks *et al*., 1991; Bacon and Hill, 1996; Hill *et al*., 1998). *Neotyphodium spp*. endophytes produce a range of alkaloids (Bush *et al*., 1997; Leuchtmann *et al*., 2000) and/or stimulate the host plants to produce alkaloids and other kinds of secondary metabolites (Bush *et al*., 1997; Ju *et al*., 1998) so that the host plants can be protected from being eaten by herbivores. Bacon (1993) found that the endophyte-fungal infected grass show tolerance to water stress through early shedding of older leaves and rolling of younger leaves in addition to low stomatal conductances and development of an enhanced osmoregulatory system.

Depending on the particular extreme environment from which they were isolated, class 2 endophyte fungi may confer benefits to their host plants through: 1) drought tolerance (Redman *et al*., 2001); 2) salt tolerance (Rodriguez *et al*., 2008b); 3) heat tolerance (Redman *et al*., 2002);

4) pathogen resistance (Redman *et al*., 1999a). Rodriguez *et al*., (2008b) have shown that class 2 endophyte fungi isolated from agricultural, coastal and geothermal habitats confer drought tolerance and more effective water utility of their host plants. A dunegrass (*Leymus mollis*) cannot survive in coastal habitats without colonization by a strain of the habitat-adapted class 2 endophyte fungus *Fusarium culmorum* that confers salt tolerance to the host plant (Rodriguez *et al*., 2008b). Redman *et al*., (2002) compared heat tolerance of infected and non-infected *Dichanthelium lanuginosum* (tropical panic grass) plants with a strain of the class 2 endophyte fungus *Curvularia protuberata*. The symbiont and host plant were able to tolerate daily root temperature regimes of 65 °C for 10 h, followed by 37 °C for 14 h or sustained 50 °C root temperatures (Redman *et al*. 2002), but the non-symbiotic plants had a maximum growth temperature of 40 °C (Redman *et al*. 2002) as did the fungus. A particular strain of *Colletotrichum magna*, another class 2 endophyte fungus, has been shown to be able to protect plants from pathogens such as *Colletotrichum*, *Fusarium*, and *Phytophthora* (Freeman and Rodriguez, 1993; Redman *et al*., 2001). Although the mechanism by which class 2 endophytic fungi benefit host plants in high-stressed environments is still unknown (Rodriguez *et al*., 2005), rapid activation of host stress response systems after symbiotic plants are exposed to stress (Redman *et al*., 1999b) and synthesis of anti-stress biochemicals by the fungus (Bacon and Hill, 1996) might be involved in the adaptation process.

1.4.2. Habitat-adapted symbiosis

Rodriguez *et al*. (2008b) suggested that endophyte fungi confer specific fitness benefits related to the habitat. Without these endophyte fungi, it can be impossible for the host plants to handle habitat-imposed abiotic stresses such as drought, high temperature and salt stress

(Rodriguez and Redman, 2008). For example, *Curvularia protuberata* can confer heat tolerance to host plants growing in Yellowstone National Park (Redman *et al*., 2002); field and laboratory assays have shown the non-symbiotic plants either shriveled or died when the root zones are heated up to 65 °C, whereas plants infected by *C. protuberata* survived. Once the fungi and the host plant grow separately, neither of them is able to tolerate temperatures above 40 °C (Redman *et al*., 2002). In addition, CThTV, a strain of virus that provides biochemical changes to the fungus, assists the fungus to confer heat tolerance to its host plant (Marquez *et al*., 2007). The symbiosis relationship in this case is actually a unique three-way symbiosis and possible contributions of viruses to other endosymbionts have not yet been investigated (Redman *et al*., 2002). Another example of habitat-specific fungal adaptation is a native dunegrass (*Leymus mollis*) on coastal beaches of Puget Sound, WA which is colonized by a fungal endophyte (*Fusarium culmorum*). A field trial showed that *L. mollis* infected by *F. culmorum* strain isolated from a coastal habitat adapted to a high salt content while *L. mollis* infected by *F. culmorum* derived from a non-coastal plant habitat could not survive there (Rodriguez *et al*., 2008b). This fact revealed that the ability to confer salt tolerance was specific to isolates from the coastal plants, indicating that the ability to confer salt tolerance is a habitat-adapted phenomenon (Rodriguez *et al*., 2008b)

Further evidence of habitat-specific adaptation are shown by some endophyte fungi isolated from geothermal plants confer a heat tolerance but not disease or salt tolerance and endophyte fungi which were isolated from coastal plants confer stress tolerance to salt but not heat or disease tolerance (Rodriguez *et al*., 2008b).

1.5. *Trichoderma harzianum*

Strains of the class 2 endophyte fungus *Trichoderma harzianum* are known to control soil-borne disease, are widely used as a biocontrol agent, and also used for industrial production of cell wall degrading enzymes (Naseby *et al*., 2000). *T. harzianum* produces an enzyme that catalyzes the breakdown of chitin, a primary component of fungal cell walls (Zeilinger *et al*., 1999), so that *T. harzianum* can successfully penetrate into the cell wall of other fungi. Penetration is followed by the release of antibiotics. The antibiotics permeate the perforated hyphae and prevent resynthesis of the host cell wall (Lorito *et al*., 1996). *T. harzianum* then cause dissolution of the cytoplasm and grow within the empty host hyphae (Inbar *et al*., 1996). *T. harzianum* has also been shown to induce metabolic changes in plants that increase the resistance of to a wide range of plant-pathogenic microorganisms and viruses (Harman *et al*., 2004). A *T. harzianum* strain, T-22 has been shown to increase the growth of plant and root development as well as solubilize various plant nutrients, such as rock phosphate, Fe^{3+}, Cu^{2+}, Mn^{4+} and Zn^{0}, that can be unavailable to plants in certain soils (Altomare *et al*., 1999), so *T. harzianum* is also considered to have evolved as an opportunistic plant symbiont (Harman *et al*., 2004).

1.6. Athabasca oil sands

1.6.1. Geographical and geological characteristics

The Athabasca oil sands in Alberta, Canada, provide an abundant source of crude oil. Although there are three large oil sand development regions in Alberta (Figure 1.6.1), the Athabasca oil sands are the largest in the world and account for >95% of the in-place resources of bitumen in North America (Hein, 2000). The Athabasca oil sands have been known for over

200 years and were originally named after the Athabasca River which passes through it and the evidence of heavy oils on the river banks (Carrigy, 1963).

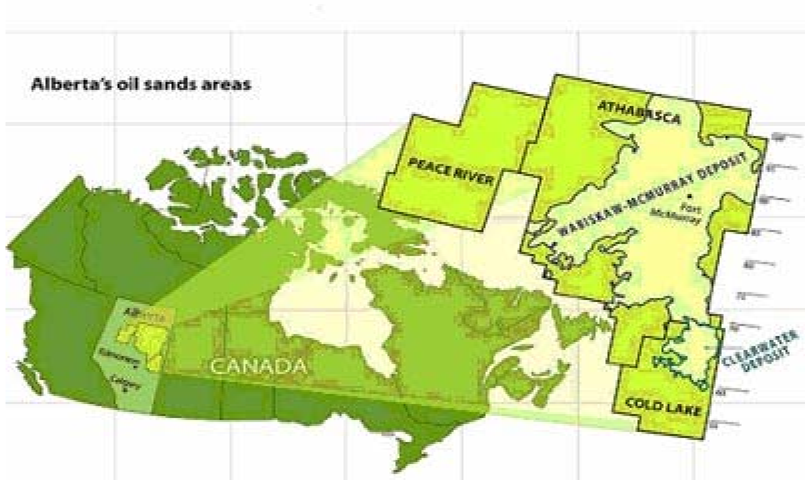

Figure 1.6.1. Map of Canada highlighting the oil sands located in Alberta. Three major oil sands deposits are located in Alberta: Peace River, Cold Lake and the largest reservoir of crude bitumen in the world, the Athabasca deposit (Radler, 2002).

The Athabasca oil sands are 75%-80% inorganic materials (sand, clay and minerals) and 3-5% water with bitumen (semisolid mixture of complex hydrocarbons derived from coal or petroleum) content ranging from 10% to about 18% (Engelhardt and Todirescu, 2005; Budgell, 2006). The Athabasca oil sand deposits are found within beds of Lower Cretaceous period (Carrigy, 1963). The origin of the oil deposits has been debated by geologists for more than fifty years with most explanations involving marine source rocks (Stanton, 2004). However, prevailing theories suggest that the oil sand's crude bitumen originated from non-marine, Carboniferous shales (359 - 300 million years ago) through the process of coalification (Stanton, 2004). Coalification is a temperature-pressure process where peat is changed to coal, and eventually with increased temperature, there is a release of water and volatiles as coal progresses to a bituminous rank (Stanton, 2004). Alberta's estimated two trillion barrels of oil sand bitumen is bordered by at least 650 billion tonnes of coal in the mountains and adjacent regions, so it is proposed that the oil originated from Lower Cretaceous sediments through the process of incomplete coalification (Stanton, 2004).

Two key characteristics of these oil sands make them economically recoverable, the area is relatively shallow and the bitumen is encapsulated by water molecules (Engelhardt and Todirescu, 2005). Escalating prices of oil and government assistance also help to make extraction economically feasible. Alberta's oil sands are one of the few oil deposits in the world with growing production and about 176 billion barrels of proven oil reserves and a total recoverable oil reserve estimated to equal almost 335 billion barrels (Engelhardt and Todirescu, 2005).

1.6.2. Oil extraction process

The mining of oil sands involves the removal of the bitumen-rich sand using open pit mining methods, which is the most efficient method of extraction when there are large shallow deposits. Another method involves processing the oil sand deposit *in situ*, where the bitumen is removed while the sand remains in place (used for oil sands that are too deep to support surface mining operations) (Budgell, 2006). For most *in situ* technologies the supply costs are about $13-$20 CAN to produce a barrel of bitumen which is marginally cheaper than the open pit mining methods (Engelhardt and Todirescu, 2005).

Oil sands are recovered from mined or *in situ* formation by similar procedures. These involve an initial emulsification of the oil with aqueous alkali, usually sodium hydroxide solution, at a pH above about 11 (Chalaturnyk *et al*., 2002). The sand and bitumen is separated by adding warm water and agitating the mixture so that coarse material is removed. The mixture is then fed into a separation tank where the sand settles to the bottom and the mixture of sand, water and bitumen (called middlings) remains suspended in the middle layer. The impure bitumen froth floats to the top layer and is removed for further processing while the sand at the bottom of the tank is pumped into tailings ponds and the middlings go through a secondary separation process. The froth is diluted with naphtha (a volatile, liquid hydrocarbon solvent) to decrease its viscosity (Hein, 2006). Water and solids remaining in the bitumen froth are removed using centrifuges and settling units. The remaining bitumen is then converted to synthetic crude oil (Budgell, 2006).

Oil sand mining methods are damaging to the environment as they involve physical disturbances such as removal of trees and animals as well as the use of local water. In addition, hydrocarbon residuals and alkali are produced as a by-product after the extraction process. Oil sand production in Canada is the leading consumer of natural gas in Canadian industry and is also the largest contributor to greenhouse gas emissions. In 1967 the first commercially viable operation began and years of exploitation since in the Athabasca oil sands have resulted in the production of massive amounts (>50 square kilometers) of tailing sands. Oil companies that use strip mining methods to extract oil are required to remediate the area to its original environmental condition once the mining is completed. However, true remediation may pose an impossible goal as some land that was once forest may never be fully restored.

1.6.3. Current remediation process in Athabasca oil sands

Tailing sands already cover an area of land greater than 50 square kilometers in Alberta. As a by-product of the oil sands extraction process, the tailings, a mixture of water, sand, silt and fine clay particles, are pumped to a settling basin after bitumen extraction. Tailings sand settles out and is used to create dykes around ponds. The remaining fine tailings gradually solidify to form mature fine tailings (Chalaturnyk *et al*., 2002). Oil companies are expending considerable effort to overcome the challenges associated with tailings disposal and ultimate site reclamation because tailings sands pose a number of environmental risks including the migration of pollutants into the groundwater system and the leakage into the surrounding soil and surface water (Price and Adam, 2008).

Traditional remediation methods of tailing sands are costly and time consuming. The cost of remediation per hectare per year is around $2000 CAN and on a 10 year remediation plan can average about $20,000 CAN (which equals roughly $2 per barrel) (Lawrence, 2008). Compared to traditional methods (mixed fertilizer with tailing sands), bioremediation such as using microorganism, fungi and green plants to reclaim tailing sands appears to be more economic and safe. According to the reclaim standard, a reclaimed growing medium should be able to support a healthy plant community (similar to that of a comparable natural area) (Eschard and Huc, 2008). Developing new vegetation on tailing sands is the key to a successful remediation.

1.6.4. Soil environment of tailing sands

A reclaimed growing medium should be able to support a healthy plant community, but due to the processes involved in bitumen extraction, tailing sands contains polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs) (Gentes *et al*., 2006) that may inhibit water absorption. In addition, mineral content is removed from the original oil sands by the extraction process (Eschard and Huc, 2008), the hot water and alkali involved in the extraction process may also kill what little soil microflora originally existed, and the high pH of tailing sands inhibits fungal growth (Kernaghan *et al*., 2002). Because of the reasons above, tailing sands are a challenging material to reclaim.

1.7. Study objectives

The main objective of this research was to observe the major fungi species associated with pioneer plants that could invade and colonize extracted oil sand sites. The second objective was to see if these fungi can confer stress tolerance in other plant species, thereby allowing the host plants inoculated with the specific fungal strains to grow in environments with similar abiotic stress factors.

1.7.1. What fungi are present in *Taraxacum* and *Sonchus* plant species?

Under the conditions with abiotic stress factors (low mineral content, hydrocarbon residual and high pH), pioneer plants such as *Taraxacum* and *Sonchus* still colonize on tailing sands. Because endophyte fungi have been shown to confer stress tolerance to host plants, endophyte fungal cultures were isolated from the samples of *Taraxacum* and *Sonchus* plants to examine the type found in the samples of extracted, remediated, and unimpacted treatments. AMF abundance inside root of *Taraxacum* and *Sonchus* plants of extracted, remediated and unimpacted treatments were also detected and compared since they have been shown to assist plants and increase mineral nutrient uptake.

1.7.2. What type of fungi is more practical in assisting the remediation process?

The application of endophyte fungi in the bioremediation process of Athabasca oil sands is expected to be more convenient and economic than using AMF. Compared to AMF, which are obligate biotrophs, class 2 endophyte fungi can be easily recovered and cultured on artificial medium. These isolates can be applied to plants directly to study whether they confer stress tolerance to their host plants. Because the tailing sands are of low nutrient content and contain a hydrocarbon residual which makes the soil of extracted treatment hydrophobic, it forms a good high-stress environment model.

1.7.3. Can endophyte fungi confer tolerance to stress on tailing sands to tomato plants?

Research has shown that class 2 endophyte fungi confer environment stress tolerance to their host plants (Rodriguez and Redman, 2007) and are of vast host range. This knowledge was applied to test whether the same endophyte fungi isolated from tailing sands pioneer plants could confer stress tolerance in a model plant, the tomato (*Lycopersicon esculentum* Mill.).

1.7.4. Endophyte species characterization

Identifying species of endophyte fungi isolated from pioneer plants which confer stress tolerance to host plants is important to see if they have been previously described in other host plants and have a vast host range. Understanding this becomes important for the potential application of the endophyte fungi in other high-stress environments besides the extracted tailing sands. Species identification also helps us to further understand the interaction between endophyte fungi and their host plants.

II. MATERIALS AND METHODS

2.1. Sample design

Five dandelion (*Taraxacum officinale*) samples and two soil samples were collected by Blair Hersikorn and Judit Smits (Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan) in July, 2007 from each of the three kinds of treatments (extracted, unimpacted and remediated) in the Athabasca oil sands. Although the geographic locations of the samples were not clear and the number of collecting sites was unknown, the samples were treated as replicates. Since whether they are really independent replicates or not were not sure, the statistics must be treated as preliminary. Samples of the extracted treatment were collected from sites that were covered by tailing sands (a by-product of oil extraction). Samples of the unimpacted treatment were collected from sites that had not been disturbed by oil extraction. Samples of the remediated treatment were collected from sites that had already been reclaimed by adding fertilizer to tailing sands artificially after oil extraction.

Figure 2.1. Photos of the three different treatments from Athabasca oil sands: unimpacted, extracted and remediated.

2.2. Soil analysis

The following preparations (except the phosphorus preparation method) were taken and modified from the Department of Soil Science (University of Saskatchewan) general protocol (de Freitas, 2007). All soils were air-dried and ground before analysis and two soil samples from each treatment were measured.

2.2.1. Measurement of soil pH

Soil pH was measured in 18 Megohm double deionized water (ddH_2O) as it most accurately reflects the pH of soil solution in the field, especially for unfertilized soils (Hendershot *et al*., 1993). A 20 g sample of air-dried soil was put into a 100 mL glass beaker. Forty mL of 18 Megohm ddH_2O was added and shaken on a rotary shaker at 142 rpm for 20 min. The suspension sat for 1 h, then was filtered through Whatman #1 filter papers into 28 mL vials. All pH readings were measured using a pH meter (VWR 8100 model), calibrated with standard solutions of pH 4.0 and pH 7.0. Three measurements of pH were taken for each of soil sample. Results were reported as the mean ± standard error of the three measurements of each sample for each treatment.

2.2.2. Measurement of soil organic carbon

Ten grams of soil samples were air dried at room temperature for 3 d. Dry soil was ground into fine powder using a mortar and pestle, then passed through a 12-mesh (approximately 2 mm) screen. Samples were weighed and approximately 0.2 g of ground soil samples were placed into a Leco CR-12 Carbon determinator (LECO Corporation, St. Joseph, MI, USA http://www.leco.com/index4.htm). Detection range of the Leco CR-12 Carbon determinator was 0.005-50%. Organic carbon was measured by combustion at 840 °C in a pure oxygen atmosphere. Soil organic C content of less than 0.2 g of soil samples can be completely oxidized within 2 min. The organic matter was converted to water and CO_2. Water was absorbed by magnesium perchlorate and the CO_2 was detected by an infrared detector. Organic carbon content was automatically calculated based on the amount of CO_2 detected by the infrared detector. Soil organic C content was reported as a percentage (%) in the soil. There were three sub-samples for each sample. For quality assurance and quality control of this test, sucrose (containing 42.1% organic carbon) was used as a certified reference material (used after every 10 tests). Results were reported as the mean ± standard error of the three measurements of each sample for each treatment.

2.2.3. Measurement of soil K, Ca, Mg, Na

Ten grams of soil samples were air dried at room temperature for 3 d. Dry soil was ground into fine powder using a mortar and pestle then passed through a 12-mesh (approximately 2 mm) screen. One gram of dried ground soil samples were placed into 50 mL plastic centrifuge tubes. Forty milliliters of 1 M ammonium acetate solution was added and the mixture was shaken on a rotary shaker at 142 rpm for 1 h. The suspension was filtered through VWR 413 filter paper into 28 mL vials and the soil solution was analyzed in an atomic absorption spectrophotometer (Spectr AA 220, VARIAN, Inc., www.varianinc.com) which is a common technique used for determining the concentration of a particular element in a given sample (Welz, 1999). Detection ranges of different elements are as following: K-0.03-2 ppm, Ca-0.01-3 ppm, Mg-0.003-1 ppm, Na-0.002-1 ppm. Wavelength of light used for measurement of K, Ca, Mg, Na content are 766.5 nm, 422.7 nm, 285.2 nm and 589 nm respecitively.

The general principle of this method rests on the fact that electrons of atoms can be promoted to higher orbitals by absorbing light of a given wavelength. Because each wavelength is specific to a particular electron transition in a particular element, it is possible to determine the amount of an element in a given sample. This technique involves the use of interchangeable filters to select light with element specific wavelengths, a flame or a furnace apparatus for volatilizing the sample, and a photon detector. Different filters are chosen that select a light that is absorbed by a specific element and the amount of light absorbed depends of the amount of element in the sample. Results were reported as mg/L in the soil. Quality control and assurance were maintained by using certified standards and appropriate method blanks

2.2.4. Measurement of soil available N

Nitrogen, pooled together in the forms of ammonium, nitrate, and nitrite ions, was extracted from the soil samples and measured as total available N content. Five grams of dried ground soil was weighed out into 100 mL glass beakers and 50 mL 2 M KCl solution was added. The mixture was shaken on rotary shaker at 142 rpm for 1 h and the suspension was filtered through VWR 413 filter paper into 28 mL vials. The N content in the soil samples (two samples of each treatment) was measured in an automatic analyzer by the ALS Laboratory Group, Saskatoon, SK. Results were reported as mg/L in the soil.

2.2.5. Measurement of Soil Extractable P

Soil P was extracted as described by McGonigle and Miller (1996). Extracts were prepared from 2 g of soil shaken for 30 min with 40 mL 0.5 M $NaHCO_3$, pH 8.5 and filtered through Whatman No. 2 paper to remove soil. The filtered extracts were sent to the ALS Laboratory Group, Saskatoon, SK. Extractable P content was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) at 882 nm there. ICP-AES is an emission spectrophotometric technique, exploiting the fact that excited electrons emit energy at a given wavelength as they return to ground state. The fundamental characteristic of this process is that each element emits energy at specific wavelengths peculiar to its chemical character. Although each element emits energy at multiple wavelengths, in the ICP-AES technique it is most common to select a single wavelength (or a very few) for a given element. The intensity of the energy emitted at the chosen wavelength is proportional to the amount (concentration) of that element in the analyzed sample. Thus, by determining which wavelengths are emitted by a sample and by determining their intensities, the analyst can quantify the elemental composition of the given sample relative to a reference standard. Results were reported as mg/L in the soil.

2.2.6. Soil hydrophobicity

The hydrophobicity assay was assessed by placing 20 μL drops of distilled water onto screened soil samples prepared as in section 2.2. Pictures were taken to record the level of hydrophobicity for different soil samples. Soils were judged to be hydrophobic if the water drop remained on the surface of the soils instead of being absorbed by the soils immediately after application.

2.3. Detection of AMF and endophytic fungi in plant tissue by wide field epifluorescence microscope and CLSM

Five dandelion (*Taraxacum officinale*) samples from each of the three treatments (extracted, unimpacted and remediated) were examined. Randomly selected lateral roots (Figure 2.3) of each dandelion samples were cut into 1 cm segments and cleared by autoclaving for 20 min in 10 % KOH. The KOH was removed with two washes in room temperature by 70 % ethanol. Cleared samples were stained for 3 h at 68 °C in 0.05 % acid fuchsin in 85 % lactic acid (Allen *et al.*, 2006; Kaminskyj, 2008). Excess stain was removed in two changes of 1:1:1 distilled water: 85% lactic acid: glycerol. Then samples were kept in the distaining solution in a 47 °C water bath overnight. The stained root samples were mounted in polyvinyl alcohol (PVAG) medium (recipe below) and the PVAG medium was polymerized overnight at 40 °C. Edges of the cover slip were sealed with transparent nail polish. Samples were observed using a wide field epifluorescence microscope, the Zeiss META 510 confocal laser scanning microscope (Allen *et al*., 2006; Kaminskyj, 2008).

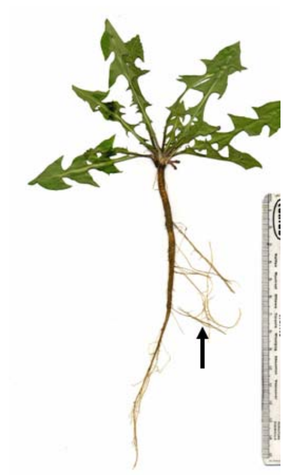

Figure 2.3. Lateral roots of dandelion (*Taraxacum officinale*). The dandelion shown in this figure was collected at the University of Saskatchewan.

The PVAG medium used in this step was modified from Brundrett *et al* (1996). PVAG medium contains 4 g polyvinyl alcohol powder (http://www.vwrcanlab.com/ JT Baker, VWR International, Edmonton, AB), 50 mL distilled water and 20 mL glycerol. This mixture was warmed to 60 °C with constant stirring and covered with a cap until it was totally dissolved (4 h to overnight). The solution will solidify and opalescent in room temperature, but it can still be re-melted with gentle heat and stirring (Kaminskyj, 2008).

Lactofushsin (LF) used for staining in this step is commonly used in the staining of plant samples to study the morphology of AMF and fungal endophytes as LF can bind to chitin, a component of most fungal walls (Bevege, 1968; Kormanik *et al*., 1980). LF has a wide range of excitation wavelengths, spanning at least 405-534 nm (blue to green) available with most epifluorescence systems (Kaminskyj, 2008).

The wide field epifluorescence microscope used for endorhizal quantitation was a Zeiss Axioplan microscope equipped a 20× N. A. 0.5 Plan Neofluar, a 40x N.A. 0.75 Plan NeoFluar, and a 63x N. A. 1.4 Plan Apochromat oil immersion objective. Images of lactofuchsin-stained material were collected through a BP546 excitation filter, FT580 dichroic mirror, and LP590 emission filter. Epifluorescence microscopy is a method where excitatory light is passed from above, through the objective and then onto the specimen instead of passing it through the specimen. Because the LF stain is a very stable fluorochrome, photobleaching is seldom a problem (Kaminskyj, 2008). Wide field epifluorescence microscopy lights up the entire field, provides an improved signal to noise ratio and is an efficient and flexible method for quantification (Kaminskyj, 2008).

A Zeiss META 510 Confocal laser scanning microscope (CLSM) (www.zeiss.com) equipped with 25× Plan NeoFluar N. A. 0.8 and 63× C-Apochromat N. A. 1.2 multi-immersion objectives was used for imaging the stained samples. Images were collected at the setting of 543 nm excitation, 9.9 % intensity of a 25 mW beam from a HeNe laser, a HFT 488/543 beam splitter, and a 604-657 nm emission filter. Fluorescence and transmitted light images were collected at the same time. CLSM provides better images of 2D and 3D objects than can be obtained with conventional optical microscopes. CLSM collects optical sections from thick specimens and uses spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus. (Allen *et al*., 2006; Ormsby *et al*., 2007; Kaminskyj, 2008)

2.4. Analysis of fungal abundance

Fungal colonization of root systems was assessed using the Multiple Quantitation Method (MQM) (Ormsby *et al*., 2007) and LF stained roots. Roots were examined by using wide field epifluorescence microscopy. Intersections were examined one field of view (1 mm) apart at 200x and 400x magnifications (Figure 2.4.1). Each intersection was assessed for the following types of endorhizal fungi: 4-6 µm wide aseptate hyphae characteristic of AM; arbuscules and vesicles associated with AM hyphae; 1-1.5 µm wide aseptate hyphae characteristic of FE; arbuscules and vesicles associated with FE; SE hyphae; and hyphal coils. Intersections not associated with any fungi were also recorded to provide an estimation of total colonization. One hypha, 2-5 hyphae and 6 hyphae per intersection were recorded as low, medium and high levels of colonization respectively. Five plant samples of each treatment were assessed, that is 52-56 intersections of each plant sample were tested. Results were expressed as mean ± standard error of the mean of the five samples and statistically analyzed by one-way ANOVA in SPSS. Significance was assessed at $P < 0.05$.

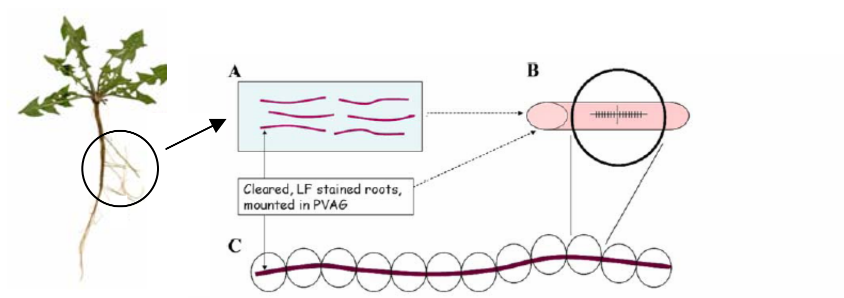

Figure 2.4.1. The scanning procedure for the Multiple Quantitation Method. Roots (A) are visualized using at 200x and 400x magnifications (B). Fungal structures that intersect the vertical line on the graticule are considered, throughout the entire focal depth of the root. (C) Intersections are evenly spaced by moving the stage by one field of view each time (Kaminskyj, 2008).

2.5. Recovering endophytic fungi from *Taraxacum* and *Sonchus* tissue

In July 2007, two dandelion (*Taraxacum officinale*) samples of each treatment (extracted, unimpacted and remediated) and four sowthistle (*Sonchus arversis*) samples of extracted treatment were collected by Dr. Ali Quoreshi (Symbiotech, Niski, AB) in the Athabasca oil sands. Plants were washed until the soil was removed, then surface sterilized in 1 % (v/v) sodium hypochorite solution for 3 min (Rodriguez *et al*., 2008b). Using aseptic technique, plants were cut into sections of 2-3 cm lengths from the roots, leaves and stems separately, plated on 10 % PDA in Petri dish and incubated for 5-7 days at 28 °C to allow for the emergence of fungi.

2.6. Purification and morphological identification of endophytic fungi strains

Endophytic fungi were recovered from plant materials (roots, leaves and primarily stems) collected from each soil type. After the primary fungi cultures had sporulated, endophytic fungi isolates were subcultured on a 10 % PDA medium (contains 3.9 g PDA powder and 12 g agar per liter) according to colony color, texture, habit, and growth rate. In order to develop a pure culture of the endophytic fungi strains from the environmental samples, a sterilized loop was used to collect a tiny amount of spores. The spores were then spread onto fresh 10 % PDA plates, which were incubated at 28 °C for 2-3 d to allow isolated single-spore colonies to emerge. The single spore colonies were then selected and subcultured. For morphological identification, a piece of agar carrying fungal tissue was cut from the pure endophytic fungi culture and a clean coverslip was placed on top of the side with fungal tissue, then incubated at room temperature in a Petri dish with distilled water. The fungi on the agar piece were allowed to grow on the coverslip until they begun to sporulate. Coverslips were mounted in lactophenol and sealed with transparent nail polish. These slides were labeled and later observed under a compound microscope for traditional morphological identification according to what has been previously described by Ellis and Ellis (1985). Pictures of fungi were captured by Sensys CCD (www. roper. com) driven by ImageManager RSI under compound microscope.

2.7. Plant colonization

Tomato (*Lycopersicon esculentum* Mill.) plants were chosen to inoculate with isolated endophytic fungi because tomato plants are a model plant system of known genome sequence and are easy to grow in a greenhouse environment. Tomato plants also have a relatively high requirement for nutrients and water, making it sensitive to environmental stress factors such as drought and low nutrition. Tomato seeds (*Lycopersicon esculentum* Mill.) obtained from plants grown in Sunshine #1 potting mixture (Sun Gro Horticulture, USA), hereafter called potting mixture, under 8 h light/16 h dark within a growth chamber at a temperature regime of 25 °C Light/ 23 °C dark (Rastogi and Sawhney, 1990) were supplied by Dr. Sawhney's lab (Department of Biology, University of Saskatchewan). Seeds were surface sterilized in 50 mL of 1% (v/v) sodium hypochlorite for 20 min with moderate agitation and rinsed with 10-20 volumes of sterile 18 MΩ water. Seeds were germinated on 10 % PDA medium and maintained in incubator for 5-7 d. To ensure this study began only with axenic plants, seedlings (ranging in size from 0.5-4 cm from cotyledons to tip of radicle) that showed no outgrowth of fungi into the surrounding medium were chosen and transplanted; any seedlings showing an outgrowth of fungi were discarded. Spores of putative endophytic fungi were harvested in 18 MΩ water by rubbing the surface of a sporulating pure culture with a sterile bent glass rod. Spore density was estimated using a hemocytometer and compound microscope (200x total magnification). The spore suspension was diluted in sterile 18 MΩ water to prepare 10^4-10^5 spore mL^{-1}. The axenic tomato seedlings were inoculated with endophytic fungi by making the seedlings immersed in 15-20 mL of spore solutions (10^4-10^5 mL^{-1}) in Petri dish for 30 min. Double-decker magenta boxes (65 mm × 65 mm × 100 mm) supplied by Dr. Bonham-Smith (Department of Biology, University of Saskatchewan) were filled with equivalent amounts (50 ± 0.5 g) of potting mixture in the upper chamber. The lower chamber was filled with 250 mL of plant food solution (Plant-Prod® All Purpose 20-20-20 Water Soluble Fertilizer, Brampton, Ontaria, www.plantprod.com). As the plant food solution was prepared as 1 g/L, it contained 200 ppm Nitrogen (N), 200 ppm Phosphoric Acid (P_2O_5) and 200 ppm soluble Potash (K_2O), in addition to 0.2 ppm Boron (B), 0.5 ppm Cu, 1 ppm Fe, 0.5 ppm Mn, 0.005 ppm Mo, 0.5 ppm Zn, and 1 ppm EDTA (http://www.plantprod.com). The solution was added with 5 mM $CaCl_2$ to stimulate endophytic fungi to colonize the plants (Rodriguez and Redman, 2007). Then the double-decker magenta boxes were sterilized by autoclaving for 22 min at 121 °C. Axenic and symbiont-inoculated seedlings were planted in the double-decker magenta boxes after they cooled down to room temperature.

2.8. Abiotic stresses assay

Low-nutrition: After two weeks growth on potting mixture, axenic and symbiont-inoculated plants were transplanted to sterile double-decker magenta boxes containing equivalent amounts (150 ± 5 g) of sterilized tailing sand. The lower chamber was filled with 250 mL of sterilized distilled water. After another two weeks growth of the plants in the greenhouse, the relative plant health of both of axenic plants and symbiont-inoculated plants was recorded by photograph and quantified according to wet and dry weight. Ten independent replicates of plant samples were taken for each treatment type.

Drought: After axenic and symbiotic plants grew on potting mixture for 2 weeks, the fluid in the lower chamber of the double-decker magenta box was poured out and the plant soils dried out over time. Soil samples were collected from the upper chamber of the double-decker magenta boxes once the tomato plants started to wilt. The soil samples were ground and weighed (W1) then dried out at 39 °C for 72 h. Dried soil samples were weighed again (W2). Moisture content of each soil sample was calculated according to the following formula: Water percentage = (W1-W2) / W1 × 100 % (Table 3.5.2). Five independent replicates of soil samples were taken for each treatment type.

2.9. Reisolation and identification of endophytic fungi from inoculated plant tissue

Plants that inoculated with isolated endophytic fungi strains were harvested and washed until potting mix or tailing sands residue was removed, then surface sterilized in 1% (v/v) sodium hypochorite solution for 3-5 min (Rodriguez *et al*., 2008b). Using aseptic technique, plants were cut into sections representing the roots, leaves and stems, plated on 10% PDA in Petri dish and incubated for 5-7 d at 28 °C to allow for the emergence of fungi.

2.10. DNA extraction

Approximately 100 µL of sand was added to a 1.5 mL microfuge tube with 500 µL of lysis buffer (contains 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). A pea-sized amount of mycelium was taken from the plate with a sterile toothpick and added to the buffer/sand mixture. The mycelium was ground with a small pestle and this mixture was vortexed for 3 min on high speed. The tube was centrifuged on high for 1 min. Then the supernatant was transfered to a new tube. After 500 μL chloroform was added to the tube, the solution was vortexed for 1 min and then centrifuged on high for 1 min. The top layer (~300 μL) was then carefully transfered to a new tube. One tenth volume of 3 M sodium acetate and 1 volume isopropanol was added to the sample and mixed well by inversion. This was left at room temperature for 15 min and then centrifuged at 13000 rpm for 15 min. The supernatant was discarded and the DNA pellet was rinsed with 500 μL 70 % ethanol. Finally, the DNA pellet was air dried for 10 min, then dissolved in 50 μL ddH_2O.

2.11. Polymerase chain reaction and sequence

Species designations were based on sequences analysis of the variable ITS1 and ITS2 sequence of rDNA. Primer pair ITS4 and ITS5 (sequences shown in Table 2.11) were used for the amplification of target sequence. PCR reactions were performed in a final volume of 50 μL containing PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP, 15 pmols of each primer, 2.5 units of Taq DNA polymerase and 100 ng of fungal genomic DNA used as template DNA.

Amplification was performed in a TECHNE Genius thermal cycler (www.techneusa.com) programmed as follows: Initial denaturation cycle at 94 °C (3 min), followed by 35 cycles of denaturation at 94 °C (20 s), annealing at 52 °C (50 s) and extension at 72 °C (1 min). The last cycle was followed by 10 min at 72 °C. For each PCR reaction, 4 μL of PCR products were separated by electrophoresis on a 1.0 % agarose gel (containing 0.5 mg L^{-1} ethidium bromide) for 30 min at 90 Voltage in 1×Tris-acetate buffer with 40 mM Tris-acetate and 1 mM EDTA , pH 8.3 (Sambrook *et al*., 1989) The PCR products were revealed under UV light. The target PCR product bands were cut from the agarose gel under UV light and purified by using QIAquick Gel

Extraction Kit (http://www1.qiagen.com/, Qiagen Inc., Stanford, Valencia, CA, USA). The concentration of the purified DNA products was measured by using UV spectrophotometer according to the absorbance at 260 nm as follows: 1) One µL DNA sample was diluted in 99 µL ddH_2O; 2) the UV disposable cuvettes were filled with 100 µL distilled water as blank to adjust the OD reading of the UV spectrophotometer to be 0; 3) the distilled water was discarded and 100 µL of diluted DNA sample was put into the dried clean cuvette; 4) the OD value of DNA sample was read at 260 nm. Because 50 µg / mL of DNA concentration (in water) has an absorbance of 1.0 at 260 nm, the concentration of the tested DNA sample can be calculated according to the formula that concentration of DNA = OD × 50 µg / mL × 100. The purified DNA fragments were sequenced at Plant Biotechnology Institution (PBI), sequencing facility, Saskatoon, SK. The sequence data were BLAST searched against the GenBank database (www.ncbi.nlm.nih.gov/BLAST/index.shtml).

To identify the *Trichoderma* spp. at species level, in Dr. Samuels' lab, PCR amplification of the internal transcribed spacer region (ITS1, 5.8 S and ITS2), a 0.65 kb section of translation elongation factor (EF-1α), 0.5 kb section calmodulin gene (cal), and 0.75 kb actin gene (act) was performed in a 50 mL reaction volume containing 5 mL of 10 x PCR buffer (New England Biolabs, Ipswitch, MA), 200 mM dNTPSs (Promega, Fitchburg Center, Wisconsin, USA), 0.2 mM of each primer, 1.25 units of *Taq* Polymerase (New England Biolabs) and 10–50 ng of template DNA. The primers used for amplification of ITS were ITS1 and ITS4 (White *et al*. 1990). For EF-1α the primers were EF1-728F (Carbone and Kohn, 1999) and TEF1 rev (Samuels *et al*., 2002). For cal the primers were CAL-228F and CAL737R (Carbone & Kohn 1999). For act the primers were Tact1 and Tact2 (Samuels *et al*., 2006). The sequences of all of the primers above are shown in Table 2.11. The program used for amplification of all the genes consisted of

3 min initial denaturation at 94°C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50°C for 30 s and extension at 72 °C for 1 min and then a final 10 min extension. The PCR products were purified using Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions then sent to sequence.

Table 2.11. Primers used in PCR

Primers	Sequence (5'-3')	Tm (°C)
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	58
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	53
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	57
EF1-728F	5'-CATCGAGAAGTTCGAGAAGG-3'	58
TEF1 rev	5'-GCCATCCTTGGAGATACCAGC-3'	61
CAL-228F	5'-GAGTTCAAGGAGGCCTTCTCCC-3'	63
CAL737R	5'-CATCTTTCTGGCCATCATGG-3'	60
Tact1	5'-TGGCACCACACCTTCTACAATGA-3'	63
Tact2	5'-TCTCCTTCTGCATACGGTCGGA-3'	64

III. RESULTS

3.1. Soil assay

The results suggest soil samples of extracted treatment were generally of the lowest nutrition content (except N and perhaps K), lowest organic C content, and highest pH compared to soil samples of remediated and unimpacted treatment in this assay (Table 3.1.1). The hydrophobicity test showed that water soaked into unimpacted soil as soon as it was applied, but water beads remained on the surface of soil samples of extracted and remediated treatments long enough for a picture to be taken (at least 5 seconds) (Figure 3.1.1). This result suggests that the soil samples of extracted and remediated treatments were relatively hydrophobic compared to the unimpacted soil. However, due to the limitation of sample size and the unclear collecting sites, these results should be treated with caution.

Table 3.1.1. Soil test data obtained from unimpacted, extracted, and remediated treatments (2 soil samples of each treatment)

	Available N (ppm)		Available P (ppm)		Calcium (ppm)	
Unimpacted	0.5	0.7	0.9	4.7	86	155.1
Extracted	0.7	0.5	0.1	0.1	4.5	4.1
Remediated	1	0.7	0.7	0.1	145.9	165
	Magnesium (ppm)		**Sodium (ppm)**		**Potassium (ppm)**	
Unimpacted	15	19.6	22.5	17.9	11	8.9
Extracted	1.1	1	12.4	13.1	7.4	3.2
Remediated	8.2	6.5	19.7	17.9	17	4.7
	Organic Carbon (%)		**pH**		**Hydrophobicity**	
Unimpacted	9.0 ± 0.2*	1.9 ± 0.2*	6.6 ± 0.1*	8.0 ± 0.1*	No	
Extracted	0.2 ± 0.0*	0.2 ± 0.0*	8.2 ± 0.2*	8.3 ± 0.1*	Yes	
Remediated	4.2 ± 0.1*	3.0 ± 0.1*	7.8 ± 0.0*	8.0 ± 0.1*	Yes	

Hydrophobicity differences were obtained qualitatively (Figure 3.1.1).
*** Values in Table 3.1.1 represent mean and standard error of 3 measurements per sample.**

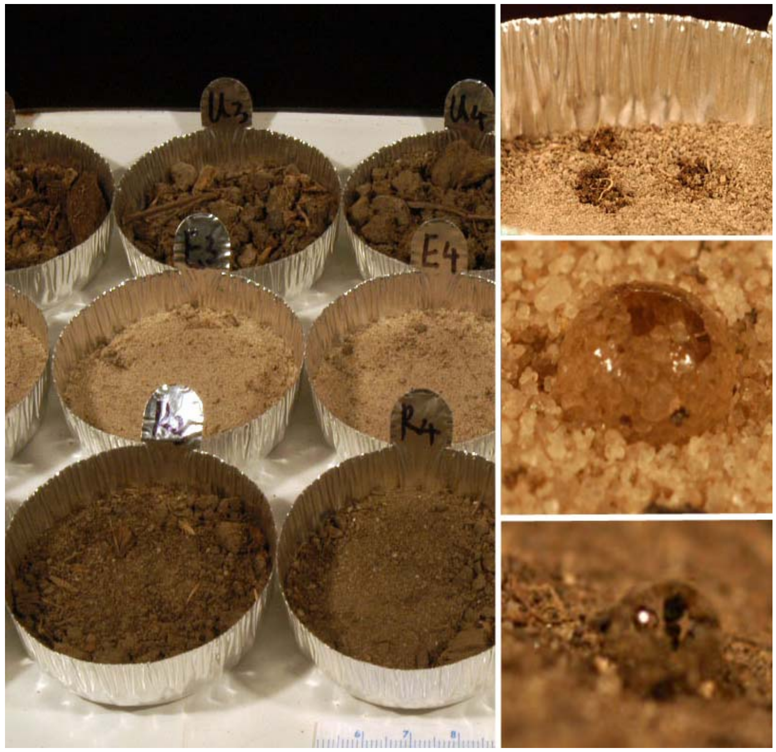

Figure 3.1.1. Hydrophobicity assay showing (from top to bottom) soil samples of unimpacted, extracted and remediated treatments. Soil samples of remediated treatment (bottom right) and extracted treatment (middle right) are relatively hydrophobic compared to soil samples of unimpacted treatment (top right).

3.2. Endorhizal fungi structures observed and relative abundance in dandelion (*Taraxacum officinale*) samples from Athabasca oil sands

Many different endorhizal fungi were found in the dandelion (*Taraxacum officinale*) samples growing upon tailing sands. AMF are characterized as having 4-6 µm wide aseptate hyphae and large spores (Figure 3.2.1 A) as well as fine-branched arbuscules (Figure 3.2.1 B). AMF hyphal coils were also observed in root samples from tailing sands as Figure 3.2.1 C shows. Fine endophytes (FE) (Figure 3.2.1 D) are characterized as the diameter of hyphae less than 2 μm. Septate endopytes (SE) (Figure 3.2.2 A) were distinguished by the septa structures in hyphae. Dark septate endophyte (DSE) (Figure 3.2.2 B), which cannot be stained by lactofuchsin, were also found in *Taraxacum officinale* root samples.

A one-way ANOVA showed there was no significant difference between the colonization percentage of AMF, SE, FE, and total fungi in *Taraxacum officinale* samples of unimpacted, extracted, and remediated treatments (Table 3.2.1).

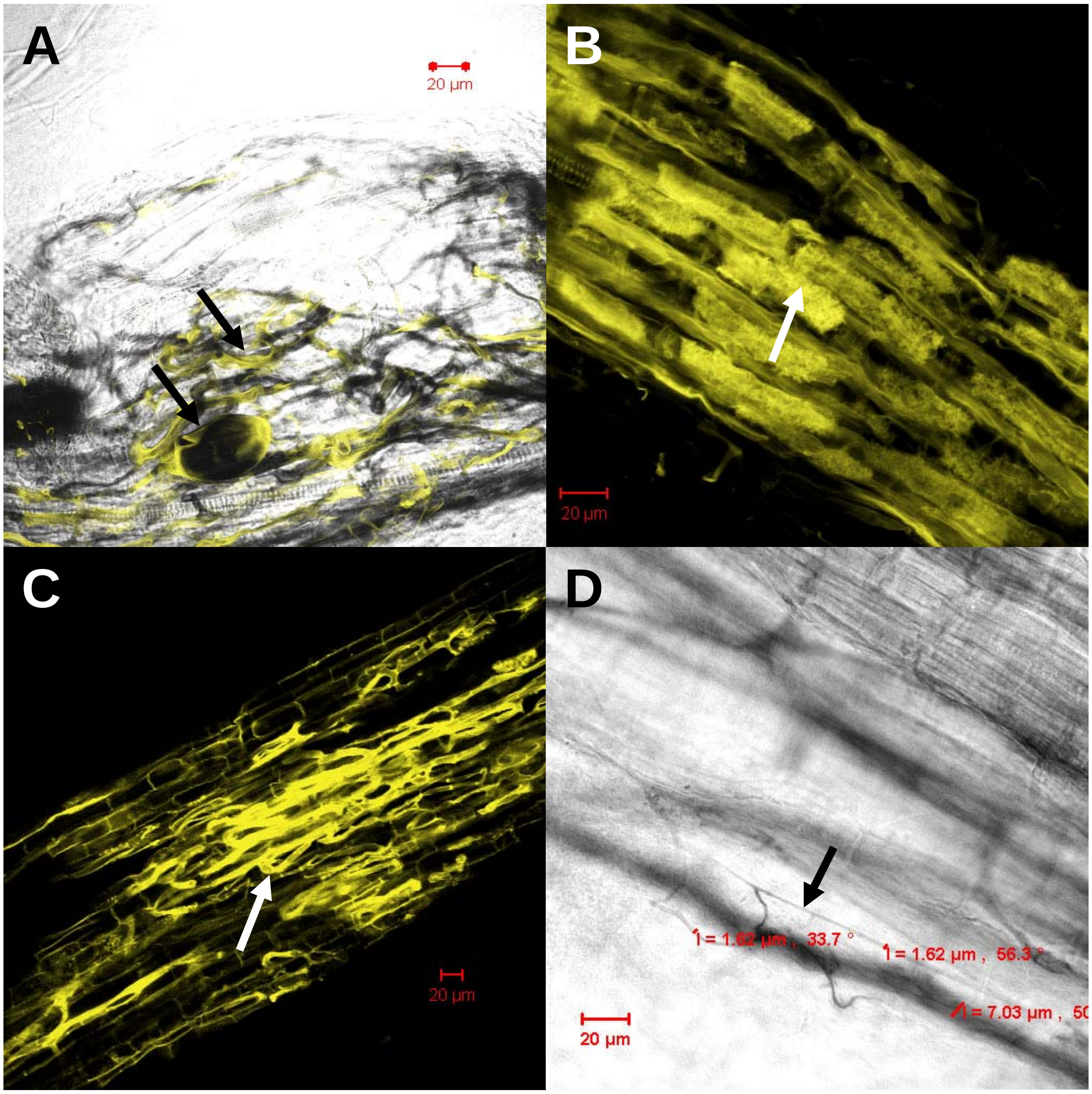

Figure 3.2.1. AMF and FE endorizal fungi in lactofuchsin-stained dandelion lateral roots from tailing sands viewed by CLSM. A-D represent AMF and FE fungi types, a natural group often associated with each other. A) AMF hyphae of 4-6 μm diameter and AMF vesicle found in dandelion lateral roots grown in tailing sands; B) Abundant arbuscules found in dandelion lateral roots grown in tailing sands; C) AMF hyphal coil observed in dandelion lateral roots grown in tailing sands; D) FE hyphae of less than 2 μm diameter found in dandelion lateral roots grown in tailing sands.

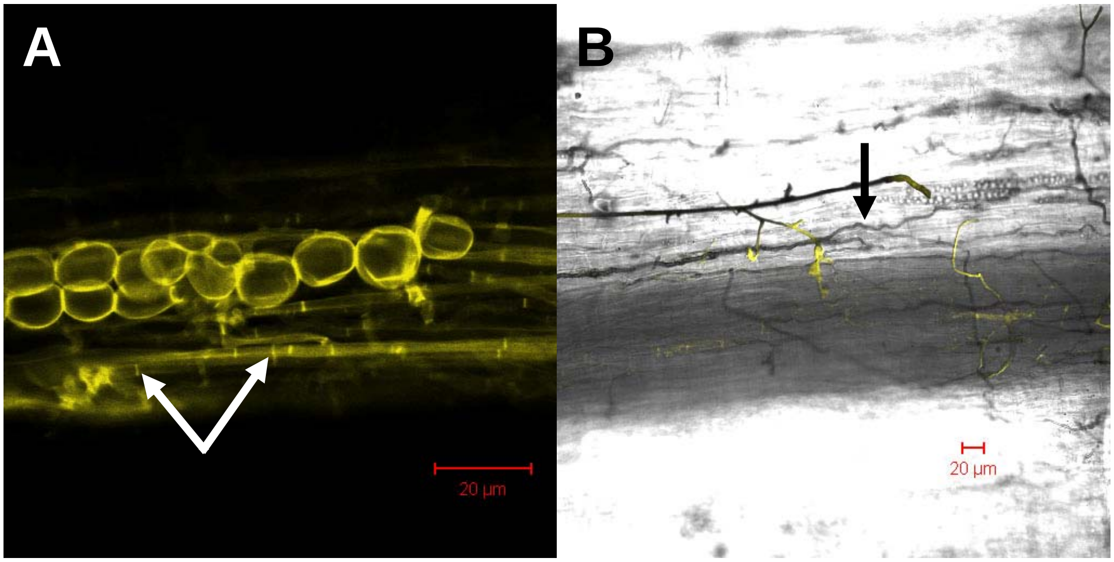

Figure 3.2.2. SE and DSE fungi in LF-stained dandelion lateral roots from tailing sands viewed by CLSM. SE and DSE fungi types form another natural fungi group. A) Septa of SE fungi found in dandelion lateral roots grown in tailing sands; B) Hyphae of DSE found in dandelion lateral roots grown in tailing sands.

Table 3.2.1. Endorhizal fungal colonization (% abundance) of dandelion roots subjected to different treatments (n=5, values represent mean of 5 samples ± standard error)

	Arbuscular mycorrihzal fungi	Septate endophyte fungi	Fine endophyte fungi	Total fungal colonization
Unimpacted	70.8±9.3	38.3±7.0	19.0±7.5	84.0±4.8
Extracted	76.4±8.5	21.3±11.4	15.8±13.9	89.4±5.6
Remediated	83.4±4.3	23.6±10.0	19.2±6.5	95.5±1.1
P-Value	0.744	0.502	0.950	0.207

Percent abundance assay of endorhizal fungi in dandelion samples of all treatments. Total fungal colonization for each sample (5 slides for each sample) was calculated as: # of intersections containing fungal structures / Total intersections x 100%. Root samples were examined microscopically (400x) using the MQM method (Ormsby *et al*., 2007).

3.3. Recovery and morphological identification of endophytic fungi from *Taraxacum* and *Sonchus* samples

Different endophytic fungi strains were represented in samples of different treatments. Identification and isolation was based on characteristics of whole colonies (Figure 3.3.1) and spore morphology (Figure 3.3.2). *Trichoderma* represented in *Taraxacum* samples of extracted treatments (Figure 3.3.1) and a *Fusarium* isolate and a *Pseudeurotium* isolate were isolated from plants growing on remediated and unimpacted soil respectively (Figure 3.3.2). *Alternaria* strains were common from all types of treatments.

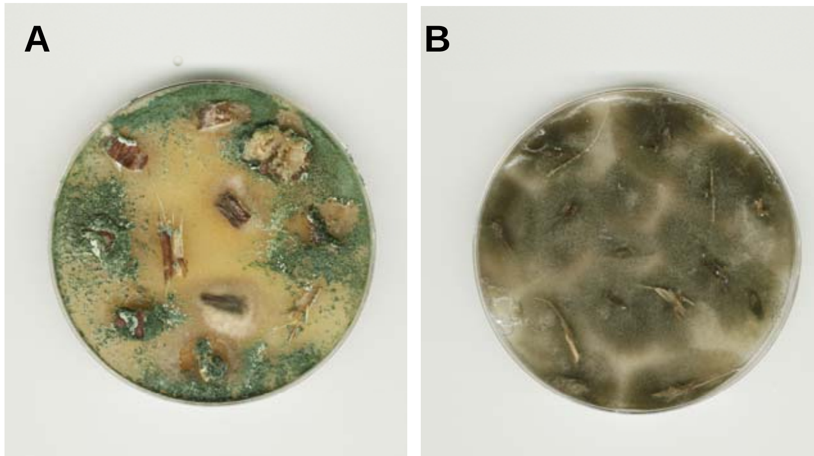

Figure 3.3.1. Fungal endophyte represented in *Taraxacum* and *Sonchus* samples from extracted treatments. A. *Trichoderma* spp isolated from *Taraxacum*; B. *Alternaria* spp isolated from *Sonchus*.

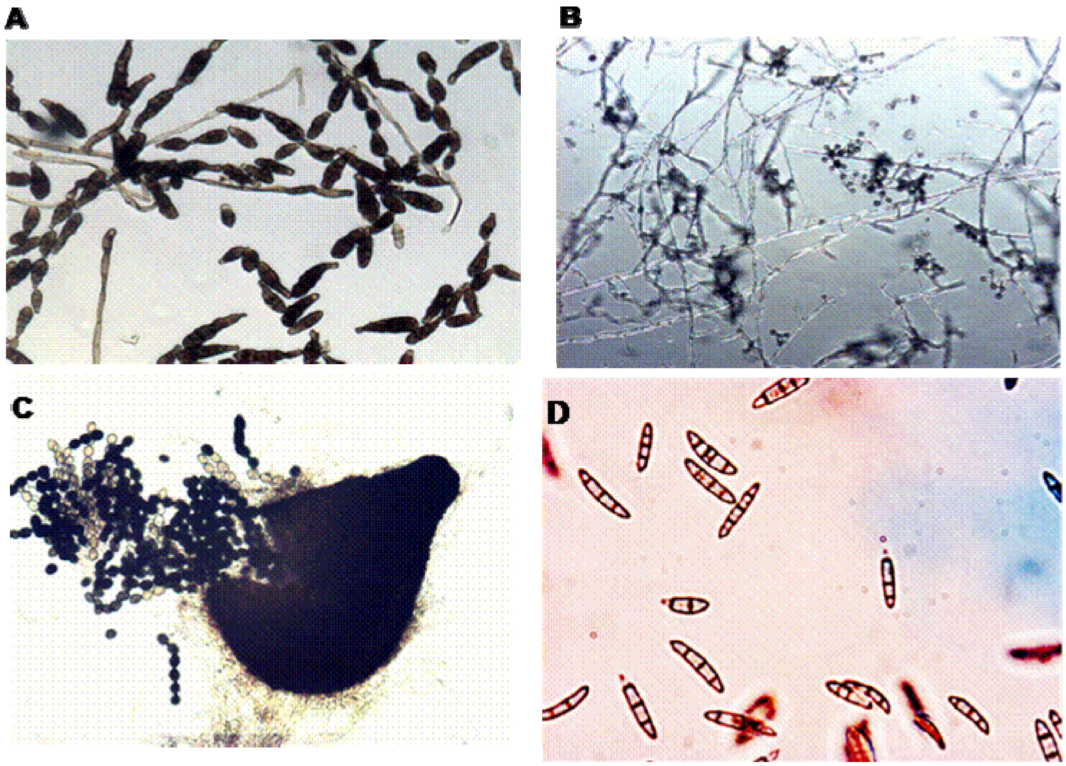

Figure 3.3.2. Endophytic fungi isolated from *Taraxacum* and *Sonchus* showing characteristic spore morphologies. A. *Alternaria* spp. represented in samples from extracted treatments; B. *Trichoderma* spp. represented in samples from extracted treatments; C. *Pseudeurotium* spp. represented in samples from unimpacted treatments D.*Fusarium* spp. represented in samples from remediated treatments.

3.4. Endophytic fungi colonization and abiotic stress treatment

Tomato plants inoculated with *Trichoderma* spores and axenic tomato plants were transplanted to tailing sands after 2 weeks growth on potting mixture (see Figure 3.4.1). After 2 weeks growth on tailing sands, tomato plants inoculated with *Trichoderma* spores grew better than axenic tomato plants. Table 3.4.1 provides quantification of this observation based on a comparison of fresh plant weight and dry plant weight. The drought test performed showed no significant difference (P = 0.155) in average moisture content at the time of wilting in soil that supported the axenic plants and the soil that supported the *Trichoderma* inoculated plants (see Table 3.4.2). Although the average moisture content was higher in soil that supported the axenic plants, the statistical result suggests that this difference is no greater than would be expected by chance alone.

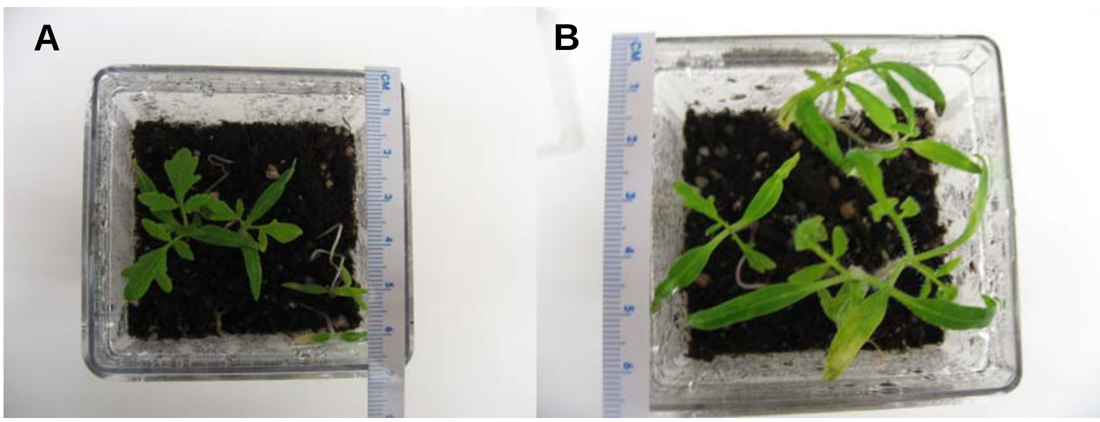

Figure 3.4.1. Photographs of tomato seedlings in potting mixture after grown under 8 h light / 16 h dark within a growth chamber at a temperature regime of 25 °C Light / 23 °C dark (Rastogi and Sawhney, 1990) for 2 weeks. A. Axenic tomato plants; B. Tomato plants inoculated with *Trichoderma* spp. spores.

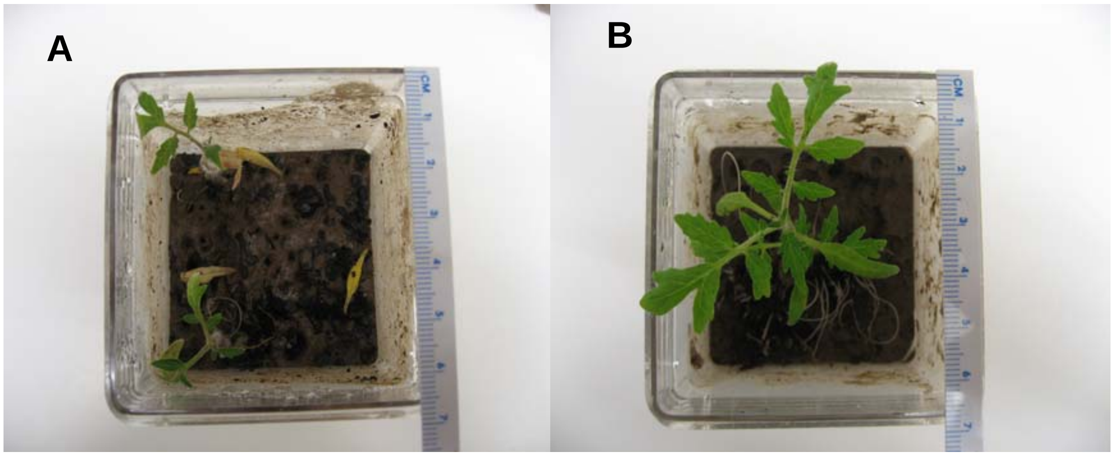

Figure 3.4.2. Photographs of axenic tomato plants (A) and tomato plants inoculated with *Trichoderma* spp. (B) after growing on tailing sands for 2 weeks. Tomato plants inoculated with *Trichoderma* spp. were growing much better and appeared to be more healthy (based on overall size and color) compared to the non-symbiotic tomato plants under the same conditions (soil, temperature, moisture and light).

Table 3.4.1. Weight (total biomass) of axenic plants and plants inoculated with *Trichoderma* after two weeks growth on tailing sands (n=10, values represent mean of 10 samples ± standard error)

	Fresh weight (g)	Dry weight (g)
Axenic tomato plants	0.196±0.063	0.014±0.002
Inoculated tomato plants	0.560±0.047	0.026±0.003
P-Value	0	0.001

Test of fresh and dry weight (total biomass) of axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. after two weeks growth on tailing sands showed tomato plants inoculated with *Trichoderma* spp. were of higher average fresh and dry weight compared to axenic tomato plants. Total biomass included roots, stems and leaves of each tomato plant. Two sample *t*-test showed a significant difference between axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. in fresh weight and dry weight ($P < 0.05$).

Table 3.4.2. Comparison of soil moisture content at start of plant wilting in drought tolerance experiment
(n=5, values represent mean of 5 samples ± standard error)

	Soil moisture content (%)
Axenic tomato plants	**22.92±2.42**
Inoculated tomato plants	**17.94±1.98**
P-value	**0.155**

Drought test of axenic tomato plants and tomato plants inoculated with *Trichoderma* spp.Two sample *t*-test showed there is no significant difference between axenic tomato plants and tomato plants inoculated with *Trichoderma* spp. in the moisture content when the plants just started to wilt ($P > 0.05$). Soil moisture content was mearsured by weight.

3.5. Detecting endophytic fungi inside of plant tissue

Using CLMS and epiflorescent microscopy, it turned out to be impossible to detect endophytic fungi associated with the inoculated tomato plant even though data presented in Section 3.6 showed they could be reisolated using microbial technique. To show what endophytic fungi look like associated with their host plants, rice seedlings were chosen based on previous evidence that showed that rice seedlings can be inoculated with endophytic fungi that confer a stress tolerance (Rodriguez *et al*., 2008b). As a monocotyledon, rice has different characteristics (such as vascular tissue organization) compared to the dicotyledon tomato plant. These differences may be responsible for the difficulty in being able to detect endophytic fungi associated with tomato plants using CLMS and epiflorescent microscopy.

Pictures of endophytic fungi in rice seedling (Figure 3.5.1) vividly show what endophytic fungi look like associated with monocotyledon plant roots. Rice seedlings inoculated with endophytic fungi from Rodriguez & Redman's lab (University of Washington, Seattle, USA) were inoculated with a *Fusarium* spp. isolated from a salt stress habitat (Figure 3.5.1 A) and a

Curvularia spp. isolated from a thermal stress habitat (Figure 3.5.1 B). These two isolates were selected for demonstration as they are believed to confer a stress tolerance, similar in function to the endophytes used to inoculate the tomato plants.

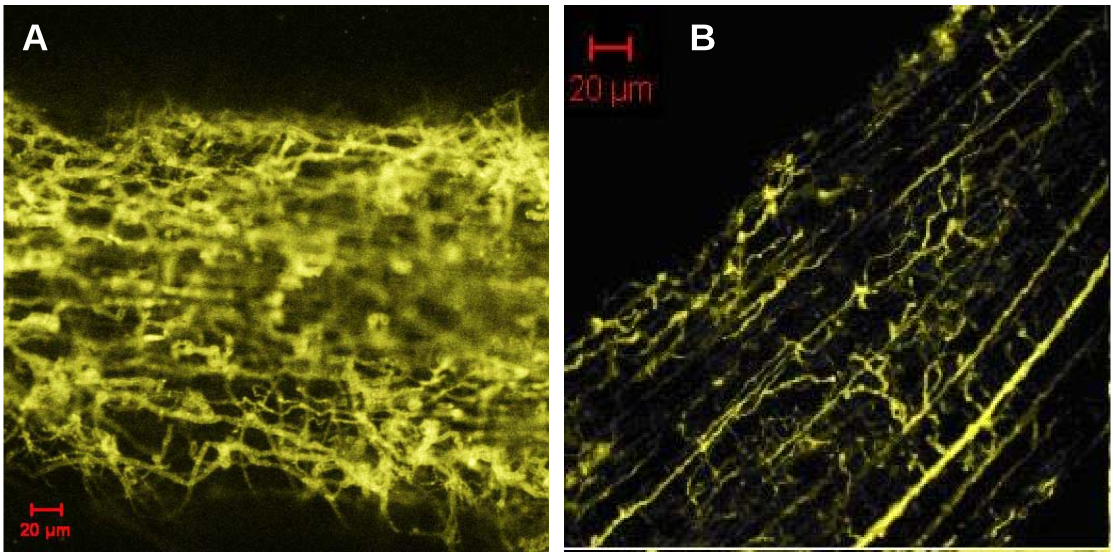

Figure 3.5.1. Confocal microscope pictures of fungi associated with roots of rice seedlings inoculated with *Fusarium* (A) and *Curvularia* (B) respectively.

3.6. Re-isolating inoculated endophytic fungi from tomato plants

Endophytic fungi colonized plants should contain a recoverable fungal strain which is identical to that used in the original inoculation. As shown in Figure 3.6.1, there were no fungi present in the axenic plant tissue (A), but there were specific endophytic fungi strains present in the inoculated plants (*Fusarium* spp. (B), *Trichoderma* spp. (C), and *Alternaria* spp. (D)). These results confirm that the plants inoculated did contain the specific endophytic fungi strains used and that there were no fungi in the axenic plants.

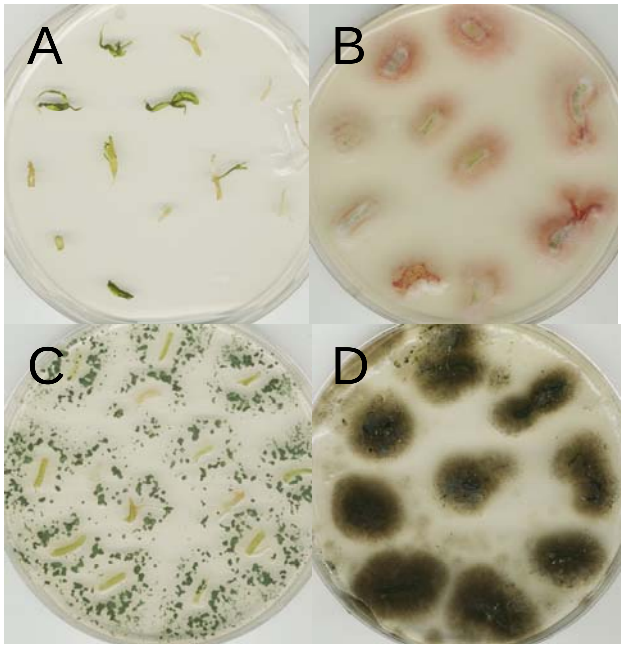

Figure 3.6.1. Pictures of fungi that had grown from axenic plants (A), and plants inoculated with *Fusarium* spp (B), *Trichoderma* spp. (C), and *Alternaria* spp. (D). Fungi were recovered on 10 % PDA medium after 5-7 days at 28 °C.

3.7. PCR products

rRNA-ITS gene fragments amplified with primers ITS4 and ITS5 from *Alternaria*, *Trichoderma* and *Fusarium* strains separated on agarose gel are shown in Figure 3.7.1. The marker used was 1000 bp and the PCR products of the endophytic fungi were around 600 bp, running on a 1% agarose gel. A *Trichoderma* strain isolated from dandelions that grew in tailing sands, which confer host plant's tolerance to tailing sands, was selected and sequenced. The DNA sequence was compared to known DNA sequence data in Genbank. The rRNA-ITS DNA sequence of *Trichoderma* strain isolated from the dandelion was a 99 % match to *Trichoderma harzianum* (*Hypocrea lixii*), *Trichoderma atroviride, Trichoderma citrinoviride* and *Trichoderma inhamatum* according to the BLAST searching result. The *Trichoderma* strain was finally identified as *Trichoderma harzianum* according to the sequence information of internal transcribed spacer region (ITS1, 5.8 S and ITS2), a 0.65 kb section of translation elongation factor (EF-1α), 0.5 kb section calmodulin gene (cal), and 0.75 kb actin gene (act) in Dr. Samuels' lab.

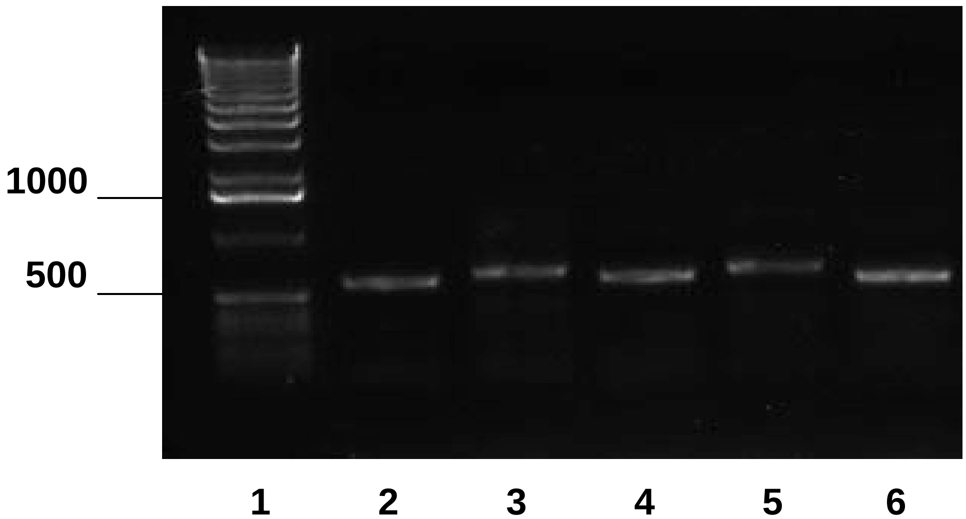

Figure 3.7.1. PCR results. 1-1kb marker, 2&4 PCR products of *Alternaria* strains, 3&5 PCR products of *Trichoderma* strains, and 6 PCR products of *Fusarium* strains.

IV. DISCUSSION

4.1. Relationship between endorhizal fungal abundance and soil environment

Plant and soil samples from three different treatments including unimpacted, extracted and remediated (Figure 2.1) were assessed in this project. Unimpacted soils analyzed here were naturally overlying the oil sands which have characteristic soil properties for the Athabasca oil sands region. Extracted soils analyzed here were tailing sands produced during the oil extraction process and had some pioneer plants such as *Taraxacum* and *Sonchus* growing on top them.

Remediated soils analyzed here were tailing sands mixed with artificial fertilizer and had plants growing on top of them. The interesting aspect is that plant samples from all of these three different treatments displayed abundant endorhizal fungal colonization (see Section 3.2.1.). Thus, perhaps, the soil microbial (or at least fungal) community appears to be consistent in quantity.

The use of hot water and alkali involved in the oil extraction process was expected to severely deplete soil microflora in tailing sands (extracted soil). However, considering the composition of the original Athabasca oil sands (about 70% inorganic materials including sand, clay, 10% water, and anywhere from 0%-18% oil) there may have been little microflora before extraction. Results showed (see Section 3.2.1.) that the abundance of fungal colonization in dandelion roots was not significantly different between the extracted, remediated and unimpacted treatments. This result does not necessarily mean that microfloras were not destroyed during the extraction process. Perhaps the original abundance of endorhizal fungi was relatively small or it is also possible that the recovery of the soil fungal community was rapid. However, no previous studies have reported whether or not this is the case. It's also possible that some fungi transmitted with plant seeds were not affected by the extraction process.

Fungal abundance in plants can be affected by diverse factors, such as host-fungus compatibility, multiple fungal species, soil properties, temperature, season and their potential combinations. My study assessed the influence of soil properties, including available soil mineral content, organic matter, hydrophobicity and pH on endorhizal fungal abundance in roots of dandelion in Athabasca oil sands. But due to the limitation of sample size and the issue of unclear collecting sites, the results cannot fully reflect the relationship between fungal abundance and soil properties.

Mycorrhizal fungi, especially AMF, have been shown to play an important role in the acquisition of P and N for host plants. Both P and N are required by mycorrhzial fungi growth (Treseder, 2004), the available P and N concentration are believed to relate to mycorrhzial abundance (Treseder and Allen, 2002). Low soil P and N concentrations has been shown to increase the hyphal growth of mycorrhizal fungi while both P and N fertilization decreases mycorrhizal abundance (Nagahashi *et al*., 1996; Dodus and Nagahashi, 2000; Treseder and Allen, 2002; Treseder, 2004). According to my soil assay results (see Section 3.1.1.), the available P concentration in soil of unimpacted treatment was higher than the available P concentration in soil of extracted and remediated treatments. Considering the artificial fertilizer added to remediated soil, the difference in P concentration in different soil samples could be due to the fact that the method used might not be sensitive enough to quantify the total available P. The higher available N concentration in soil of remediated treatment relative to extracted and unimpacted treatments may be due to fertilization in the remediated sites. However, there were no significant differences between these treatments in terms of fungal abundance.

Nogueira and Cardoso (2006, 2007) reported that growth of soybean (*Glycine max*) and rangpur lime (*Citrus limonia*) was not affected when the soil P concentration in the soil-sand mixture (three parts of sandy soil (Typic Quartzipsamments) and one part washed sand) is between 50-100 ppm. Plants respond to mycorrhiza positively and negatively when the available soil P concentration is below 50 ppm and above 100 ppm, respectively. High mycorrhizal abundance in all three treatments may result from available soil P concentration in the extracted, remediated and unimpacted soils being lower than 50 ppm (see Section 3.1.1). Also, the difference in available soil P concentration between the three treatments may not be big enough to make a difference in the mycorrhizal colonization rate in host plants from different treatments.

Responses of mycorrhizal abundance to N concentration were reported to be positive (Lussenhop *et al*., 1998), negative (Klironomos *et al*., 1998) or not significant (Bethlenfalvay *et al*., 1999). The inconsistent effect of N concentration may be related to the variation in responses among mycorrhizal groups or among groups of plant and fungi combinations (Treseder and Allen, 2000). Previous research has shown that composition of AMF community is shifted with available soil N concentration. For example, Eom *et al*., (1999) found the abundance of spores from *Gigaspora gigantea* and *Glomus mosseae* increased with N fertilization. In contrast, spores from *Entrophospora infrequens* declined significantly. The differences in available N concentrations in soil samples of different treatments may change the composition of AMF community but not total abundance of AMF.

The extracted soils were shown to contain the lowest Ca and Na in my assay, while the fungal abundance is still similar to the samples of unimpacted and remediated treatments. Although low soil exchangeable Ca concentration may affect the abundance of AMF colonization in host plants in the way of causing premature root senescence which would have negative effects on AMF sporulation and colonization (Jarstfer *et al*., 1998), there was no previous research that reported exchangeable Ca and Na concentrations directly controlling the abundance of mycorrhizal fungi. Rodríguez-Echeverria *et al*., (2008) also did not find any relationship between AMF colonization and concentration of soil exchangeable Ca and Na after they examined AMF abundance and soil properties in six locations of a European coast.

AMF have been observed to concentrate hyphae in organic matter (Hepper and Warner, 1983) and organic matter is important in providing a substrate for the development of some kinds of mycorrihizal fungi (Allen *et al*., 1993). AMF can form soil structural units (aggregates) through the combined action of AMF hyphae, glomalin (a hyphal wall component) or even

AMF-associated microbes (Rillig *et al*., 2005). Aggregates can provide resistance against erosion and benefit the AMF by reducing erosion-related host plant losses (Rillig, 2004b). Although the organic carbon content of soil samples of the three treatments varied from lowest in the extracted samples and highest in unimpacted samples in my soil assay, the abundance of mycorrhizal colonization are not significantly different among the three treatments (see Tables 3.1.1. and 3.2.1.). Considering the limitation of sample size and the issue of unclear collecting sites, perhaps a larger sample size and corresponding collecting sites will provide more convincing evidence to show the relationship between soil organic matter and mycorrhizal fungal abundance. However, since mycorrhizal fungal abundance results from different factors, the combination of other soil properties should also be considered in addition to organic carbon content.

The occurrence of AMF has been shown to correlate with soil pH in some previous studies (Tadych and Blaszkowski, 2000). But Ursic *et al*. (1997) found that the density of AMF in *Pinus strobus* seedlings was not related to the soil pH. Porter *et al*. (1987) reported that soil pH affects the preference of certain fungi to colonize plant roots. *Acaulospora leavis* is found more in acid soil (~ pH 4.7) than in neutral soil and germination of *Acaulospora leavis* spores requires a pH > 6 (Porter *et al*., 1987). Three strains of *Glomus* were found to be more adaptable in soil of pH ranging from 5.5-8.4 (Porter *et al*., 1987). Although in this study the extracted soil samples had a higher pH than the unimpacted and remediated soil samples, there were no apparent differences of mycorrhizal abundance in dandelion roots growing in soil of different pH (see Tables 3.1.1. and 3.2.1.). It is possible that no influence of soil pH on mycorrhizal fungal abundance in this study was found because of the different responses of different mycorrhizal fungi groups to soil pH as well as the dandelion-fungi combination. It is also possible that the pH difference among these three kinds of soil samples is too minor to influence mycorrhizal fungal abundance.

Many studies have reported that drought stress increases the colonization abundance of mycorrhizal fungi. For example, 43 flowering plants growing on a fallow agricultural site in Germany were examined, 40 of them were heavily infected by AMF in a low soil moisture habitat, while only 29 of them were heavily infected in a comparable but high soil moisture habitat (Kuhn, 1991; Kuhn *et al*., 1991). In my study, soil samples of remediated and extracted treatments were shown to be relatively hydrophobic (see Figure 3.1.1.). This result implies that the soil moisture may be lower in these two kinds of soil compared to in the unimpacted soil. But there was no apparent difference in mycorrhizal colonization abundance between all three treatments. Spore germination can be increased (Douds and Schenck, 1991), decreased (Tommerup, 1984; Estaun, 1990; Douds and Schenck, 1991) or unaffected (Douds and Schenck, 1991) by soil drying depending on the species. For example, in Douds and Schenck's study (1991), *Acculosporu longula* spores were observed to increase germination rate as soil water content increases. *Glomus intramdices* were observed to decrease germination rate as soil water content increases, while the germination rate of *Gigaspora margarita* spores were not significantly affected by soil water content (Douds and Schenck, 1991). The similarity of mycorrhizal abundance in my study could be contributed to different compositions of mycorrhizal community in samples from the different treatments. In addition, the influence of soil moisture content on mycorrhizal fungal abundance may be too minor to be observed.

Few studies about the relationship between soil properties and endophytic fungal abundance have been done before. Tian *et al*. (2003) reported acidic soil is more ideal for the growth and colonization of class 2 endophyte fungi such as *Fusarium* spp. in rice compared to alkali soil.

Blodgett *et al*. (2006) showed that the abundance of class 2 endophytic fungi such as *Alternaria tenuissima*-like group and *Fusarium* species associated with *Amaranthus hybridus* were significantly influenced by soil modifications and watering. Rodriguez and Redman (2007) also reported that environmental conditions dictate class 2 endophyte population dynamics in plants co-colonized with endophytes that confer different stress tolerances. However, in my study, the abundance of endophytic fungi (SE) was not significantly different between all three treatments (see Table 3.2.1). For my study, the fungal abundance assay only included fungi colonization in roots, although the distribution of endophytic fungi in below and above ground tissues is different (Tian *et al*., 2003). The similarity of endophytic fungal abundance may result from the bias of root detection only without considering fungal colonization in the above ground tissue. In addition, whether the SE observed inside of the dandelion roots was class 2 endophyte fungi is not yet clear, as class 2 endophyte fungi isolated from dandelion and sowthistle were not detected in inoculated tomato plants using CLMS (see Section 3.5). However, SE in dandelion roots could be observed using CLMS. If the SE observed were not class 2 endophytes, perhaps factors that affect the abundance of class 2 endophyte fungi may not influence the density of SE. Also, a specific stress (i.e. drought and low mineral content in tailing sands) only increases the population of class 2 endophytic fungi which are adapted to the stress (Rodriguez and Redman, 2007) but may not influence the total endophytic fungal abundance.

All in all, a larger sample size and corresponding accurate collecting site information are necessary for studying the relationship between different endorhizal fungal abundance and soil environment in the future.

4.2. Limitation of methods involved in endophytic fungal recovery and morphological identification

As previously discussed by several authors (Bills and Polishook 1992, 1994; Livsey 1994; Collado *et al*., 1996; Polishook *et al*., 1996), the method used for isolation determines the frequency and species distribution of fungi recovered from a certain host. Bills and Polishook (1992) found a mixture of 1% malt extract and 0.2% yeast extract provides the highest species richness for isolations of endophytic fungi from twigs and leaves of *Chamaecyparis thyoides*. Santamara and Diez (2005) reported that incubation in moist chambers resulted in significantly more fungal isolates and much higher species richness compared to isolation on PDA when they tried to recover fungi from *Populus tremula* tissue. Cao *et al*. (2000) reported water agar seemed to be a better medium than PDA for isolating endophytic fungi from *Musa acuminata* leaves and roots. Although PDA has often been cited as a common, unselective medium used for endophytic fungal recovery (Camacho *et al*., 1997; Rodriguez and Redman, 2008), using PDA may result in underestimating the actual number of endophytic fungal species in plant samples (Santamara and Diez, 2005). Class 2 endophyte fungi may have different nutritional strategies than other groups and rich medium might overwhelm the growth of class 2 endophyte fungi. Therefore, high species richness may result from species of fungi from other endophyte groups.

In my study, 10% PDA at pH 5.0 was the only culture medium used in endophytic fungal recovery. The nutrient concentration and pH at these levels may be more ideal for some strains of endophytic fungi. Therefore, some strains of endophytic fungi cultured on this PDA may have a competitive advantage in terms of growth and resource acquisition. Some fungi may even inhibit the growth of other endophytic fungi through excreting secondary metabolic products, providing another competitive advantage. Involving more kinds of culture medium for isolating endophytic fungi from plant tissue in future studies may provide a more accurate representation of endophytic fungal species richness.

In this study, plant tissue segments placed on PDA medium were incubated for 5-7 days at 28 °C to allow for the emergence of fungi. Since the growth rate of endophytic fungi can be different, perhaps the incubation time may not be long enough to allow some endophytic fungi to emerge. Future studies may address this issue by prolonging the incubation period. Also, reducing the culture temperature may result with more fungal colonies represented since the temperature of natural soil is lower.

4.3. Possible mechanisms of habitat-specific stress tolerance to host plants conferred by class 2 endophytic fungi

Class 2 endophytic fungi may significantly affect the eco-physiology of different habitats through the rapid adaptation of plants to high-stressed habitats (Rodriguez *et al*., 2008a). Class 2 endophytic fungi are unique for their ability to confer habitat-specific stress tolerance to host plants (Rodriguez *et al*., 2008b). Previous research has shown that class 2 endophytic fungi increase host shoot and/or root biomass by the induction of plant hormones by the host or biosynthesis of plant hormones by the fungi (Tudzynski and Sharon, 2002). Class 2 endophytic fungi may confer disease tolerance to host plants by producing secondary metabolites (Schulz *et al*., 1999), through fungal parasitism (Samuels *et al*., 2000) and induction of systemic resistance (Vu *et al*., 2006) or because of an inability of pathogens to compete with endophytic fungi for resources or niche space (Rodriguez *et al*., 2008a). The cellular mechanisms involved in stress tolerance and growth improvement are not yet clearly known (Rodriguez *et al*., 2008a). Redman *et al*. (1999b) reported endophyte-colonized plants activate host defenses more quickly compared to non-symbiotic plants when exposed to virulent pathogens, while without pathogen exposure, plants colonized with class 2 endophytic fungi do not activate host defenses. This indicated that certain biochemical processes are involved in symbiotically conferred stress tolerance (Rodriguez *et al*., 2008a).

Some similar plant responses have been found when plants were exposed to heat, drought and salt stress, including increased osmolyte production, altered water relations, production of signaling molecules such as abscisic acid (ABA) and the generation of reactive oxygen species (ROS) (Bohnert *et al*., 1995; Bray, 1997; Wang *et al*., 2003; Apel and Hirt, 2004). Rodriguez *et al*., (2008a) reported there is a strong correlation between ROS susceptibility and stress tolerance in endophytic fungi colonized plants. For example, non-symbiotic plant tissues bleached white and class 2 endophytic fungi (*Fusarium culmorum*) colonized plant tissues remained green after exposed to abiotic stress and then paraquat (a herbicide that induces ROS production and subsequent photobleaching of chlorophyll; Vaughn and Duke, 1983) (Rodriguez *et al*., 2008a). *Fusarium culmorum* confers salt but not heat tolerance and only protects plants exposed to salt stress from photobleaching but not plants exposed to heat stress. Meanwhile, *Curvularia protuberata*, which confers heat but not salt tolerance, only protects plants exposed to heat stress from photobleaching but not plants exposed to salt stress (Rodriguez *et al*., 2008b). This result indicates ROS protection in endophytic fungi colonized plants occurs in a habitat-specific manner (Rodriguez and Redman, 2007) and may result in class 2 endophytic fungi confering stress tolerance to host plants in a habitat-specific manner.

Many previous studies have shown that drought tolerance in plants is usually related to increased osmotic potential (Bohnert *et al*., 1995; Bray, 1997; Wang *et al*., 2003). However, Rodriguez *et al*., (2008b) reported that non-symbiotic plants wilt much earlier (6-10 days) than class 2 endophytic fungi symbiotic plants even though the osmotic potential does increase in non-symbiotic plants. Symbiotic plants were shown to consume 30-50% less water than non-symbiotic plants (Rodriguez *et al*., 2008b). In my study, tomato plants inoculated with the class 2 endophyte fungi (*Trichoderma harzianum*) started to wilt at lower average water content in soil compare to axenic tomato plants (see Table 3.4.2). These results suggest that class 2 endophytic fungi conferred drought tolerance through more effective water use instead of correlating with an increase in osmotic potential.

In my study, the class 2 endophyte, a strain of *Trichoderma harzianum*, was isolated from plants growing on tailing sands and found to confer low nutrient and drought tolerance to artificially inoculated host plants (see Section 3.4). Altomare *et al*. (1999) reported a *Trichoderma harzianum* Rifai 1295-22 (T-22) strain that could solubilize *in vitro* insoluble or sparingly soluble minerals such as MnO_2, metallic zinc, and a rock phosphate (mostly calcium phosphate) through acidification, producing chelating metabolites and redox activity. T-22 has also been shown to enhance nitrogen use efficiency in corn (Harman, 2000). Considering the relatively low nutrient content in tailing sands, the *Trichoderma harzianum* strain isolated from tailing sands samples may increase the available mineral concentration to supply enough essential nutrients for host plant growth. Harman (2000) reported that roots colonized by T-22 reduced the sensitivity of crops to drought stress and resulted in enhanced root development. The *Trichoderma harzianum* strain isolated from tailing sands samples may confer drought tolerance through more effective water use as well as enhance the development of root growth. The latter may increase both of water and nutrient acquisition in symbiont inoculated plants relative to anexic plants that may have a less developed root system. Lynch (Patent No. 7273552) found by providing a compatible rhizosphere, a strain of *T. harzianum* are capable of protecting the plant as well as being able to detoxify their environment by processing larger amounts of contaminant through higher metabolic rates, the *T. harzianum* isolated from plants growing on tailing sands may confer the tolerance of the host plant to PAH residue in tailing sands in the similar methods.

4.4. Molecular identification of *Trichoderma* strain isolated from tailing sands

In my study, ITS1 and ITS2 regions of the *Trichoderma* strain were amplified and sequenced. The sequence information obtained from ITS1 and ITS2 regions were not specific enough to identify the *Trichoderma* strain to species level (see Section 3.8.). Many studies have made heavy use of ITS1 and/or ITS2 (Kuhls *et al*., 1997; Kindermann *et al*., 1998; Lieckfeldt *et al*., 1998; 2001; Dodd *et al*., 2000) since this gene cluster is present for > 90 copies per genome and thus can be easily amplified. However, using ITS1 and ITS2 becomes discredited in identifying some fungi such as the closely related *Fusarium* and in plants that have been shown to contain paralogous copies of the parts of the rDNA gene cluster (O'Donnell, 1992; Buckler *et al*., 1997; O'Donnell *et al*., 1998; Lieckfeldt and Seifert, 2000). ITS was thought to provide poor resolution for closely related species (Druzhinina *et al*., 2005). In addition, the use of ITS1 and ITS2 provides only poor phylogenetic resolution in some clades, especially *Pachybasium*, which includes *T. hamatum, T. harzianum, T. virens, T. viride and T. koningii* (Kullnig-Gradinger *et al*., 2002; Chaverri *et al*., 2003a). The sequence information of ITS1 and ITS2 fragments were submitted to a BLAST and the best match to species was accepted. But the GenBank databases contain many ITS sequences of *Trichoderma* isolates which had been incorrectly identified and thus occur under a false name (Druzhinina and Kubicek, 2005). For example, Kullnig *et al*. (2001) reidentified several strains as *Trichoderma harzianum* that had been reported in the literature under different names. Therefore, the BLAST result may mislead the identification of *Trichoderma* based on the ITS sequence information.

The translation elongation factor 1 alpha (EF-1α) loci consist of several relatively large and variable introns and exons and in total exceed 2 kb in length. Different fragments of EF-1α were found to be the most promising loci compared to the 11 tested loci/fragments (Druzhinina *et al*., 2005). Because the sequence information of ITS1 and ITS2 region is not specific enough for *Trichoderma* identification, the sequence information of the section calmodulin gene (cal), actin gene (act) and EF-1α, were performed as a complement of ITS sequence information (Samuels *et al*., 2006) in Dr. Samuels' lab. Therefore, in my study the *Trichoderma* strain that conferred a stress tolerance to host plants was identified as *Trichoderma harzianum.* However, *Trichoderma harzianum* is usually a complex of morphologically cryptic species (Chaverri *et al*., 2003b). Therefore, a future study involving the use of RAPD or AFLP techniques may be needed to confirm whether the isolation I present is a single or multiple *Trichoderma* strain.

4.5. Future research directions

Field trials in a natural tailing sands environment are necessary to confirm and optimize use of the *T. harzianum* strain that confer host plants' tolerance to tailing sands in small-scaled greenhouse experiments. More host plants such as some weedy species will be used and compared with tomato plants as they are more suitable for practicle remediation use in tailing sands.

Some research has shown naphthenic acids (NAs), a kind of toxic organic acid compounds formed during the caustic extraction process, are phytotoxic and cause cell death in the plant root epidermis as well as change the chemistry of parenchyma cells in the root pith

(Armstrong, 2008). To figure out whether the long-term *T. harzianum* strain treatment can impact the soil environment and naphthenic acid degradation in tailing sands will be a future goal.

The identification and description of other possible endophytes that assist pioneer plant growth in the Athabasca oil sands may also be important in assisting current remediation methods. Based on unique class 2 endophytes which establish habitat-specific symbiosis with their host plants, similar methods as described in this study can be used in other high-stress environments. This could potentially result in transferring the tolerance of the particular abiotic or biotic stress factors confered by the class 2 endophyte to other host plants.

V. APPENDIX

Alternaria spp. isolated from plant samples from unimpacted, extracted and remediated treatments, a *Pseudeurotium* spp. isolated from plant samples from unimpacted treatments and a *Fusarium* spp. isolated from plant samples from remediated treatments were also used to inoculate axenic tomato seedlings. However, all of these fungal strains were harmful to their host tomato plants, resulting in either visably weakened mature plants or death of the host plant. As the investigation into harmful fungal strains was not the purpose of study, these results were not included and a further analysis was not performed.

VI. REFERENCES

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature (London) *435,* 824-827.

Allen, N., Nordlander, M., McGonigle, T., Basinger, J., and Kaminskyj, S. (2006). Arbuscular mycorrhizae on Axel Heiberg Island (80 degrees N) and at Saskatoon (52 degrees N) Canada. Canadian Journal of Botany *84,* 1094-1100.

Altomare, C., Norvell, W.A., Bjorkman, T., and Harman, G.E. (1999). Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. Appl. Environ. Microbiol. *65,* 2926-2933.

Apel, K., and Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology *55,* 373-399.

Armstrong, S., (2008). Dissipation and Phytotoxicity of Oil Sands Naphthenic Acids in Wetland Plants. Thesis (PhD) University of Saskatchewan.

Arnold, A.E., and Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology *88,* 541-549.

Auge, R.M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza *11,* 3-42.

Bacon, C.W. (1993). Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue. Agriculture Ecosystems & Environment *44,* 123-141.

Bacon, C.W., and Hill, N.S. (1996). Symptomless grass endophytes: Products of coevolutionary symbioses and their role in the ecological adaptations of grasses. Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology, and Evolution 155-178.

Bago, B., and Becard, G. (2002). Bases of the obligate biotrophy of arbuscular mycorrhizal fungi. Mycorrhizal Technology in Agriculture: From Genes to Bioproducts 33-48.

Bakker, F.T., Olsen, J.L., Stam, W.T., and Vandenhoek, C. (1992). Nuclear ribosomal DNA internal transcribed spacer regions (ITS1 and ITS2) define discrete biogeographic groups in *Cladophora Albida* (Chlorophyta). J. Phycol. *28,* 839-845.

Bentivenga, S.P., and Morton, J.B. (1994). Systematics of glomalean endomycorrhizal fungi: Current views and future directions. Mycorrhizae and Plant Health 283-308.

Berbee, M.L., and Taylor, J.W. (2007). Rhynie chert: a window into a lost world of complex plant-fungus interactions. New Phytol. *174,* 475-479.

Bethlenfalvay, G.J., Cantrell, I.C., Mihara, K.L., and Schreiner, R.P. (1999). Relationships between soil aggregation and mycorrhizae as influenced by soil biota and nitrogen nutrition. Biol. Fertility Soils *28,* 356-363.

Bevege, D.I. (1968). A rapid technique for clearing tannins and staining intact roots for detection of mycorrhizas caused by *endogone* spp and some records of infection in Australasian plants. Transactions of the British Mycological Society *51,* 808-810.

Bever, J.D., Schultz, P.A., Pringle, A., and Morton, J.B. (2001). Arbuscular mycorrhizal fungi: More diverse than meets the eye, and the ecological tale of why. Bioscience *51,* 923-931.

Biermann, B., and Linderman, R.G. (1983). Use of vesicular arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytol. *95,* 97-105.

Bills, G.F., Polishook, J.D., (1992). Recovery of endophytic fungi from *Chamaecyparis thyoides*. Sydowia *44*, 1-12.

Bills, G. F., Polishook, J. D., (1994). Microfungi from decaying leaves of *Heliconia mariae (Heliconiaceae)*. Brenesia 41-42, 27-43.

Blodgett, J.T., Swart, W.J., Louw, S.V., and Weeks, W.J. (2007). Soil amendments and watering influence the incidence of endophytic fungi in *Amaranthus hybridus* in South Africa. Applied Soil Ecology *35,* 311-318.

Bohnert, H.J., Nelson, D.E., and Jensen, R.G. (1995). Adaptations to environmental stresses. Plant Cell *7,* 1099-1111.

Bois, G., Bertrand, A., Piche, Y., Fung, M., and Khasa, D.P. (2006). Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. Mycorrhiza *16,* 99-109.

Bolan, N.S. (1991). A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil *134,* 189-208.

Bonfante, P., and Perotto, S. (1995). Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol. *130,* 3-21.

Bray, E.A. (1997). Plant responses to water deficit. Trends Plant Sci. *2,* 48-54.

Bridge, P., and Spooner, B. (2002). Interactions in the root environment - An integrated approach: Proceedings of the millennium conference on rhizosphere interactions, IACR-Rothamsted, UK, 10-12 April 2001 147-154.

Brundrett, M., and Kendrick, B. (1990). The roots and mycorrhizas of herbaceous woodland plants .2. Structural Aspects of Morphology. New Phytol. *114,* 469-479.

Brundrett, M.C. (2006). Understanding the roles of multifunctional mycorrhizal and endophytic fungi. In: Schulz BJE, Boyle CJC, Sieber T.N., eds. Microbial root endophytes. Berlin: Springer-Verlag, 281–293.

Buchan, A., Newell, S.Y., Moreta, J.I.L., and Moran, M.A. (2002). Analysis of internal transcribed spacer (ITS) regions of rRNA genes in fungal communities in a southeastern US salt marsh. Microb. Ecol. *43,* 329-340.

Buckler, E.S., Ippolito, A., and Holtsford, T.P. (1997). The evolution of ribosomal DNA: Divergent paralogues and phylogenetic implications. Genetics *145,* 821-832.

Budgell, P. (2006). State of the industry review: The Canadian oil sands industry. Government of Alberta, Canada.

Bush, L.P., Wilkinson, H.H., and Schardl, C.L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol. *114,* 1-7.

Camacho, F.J., Gernandt, D.S., Liston, A., Stone, J.K., and Klein, A.S. (1997). Endophytic fungal DNA, the source of contamination in spruce needle DNA. Mol. Ecol. *6,* 983-987.

Cao, L.X., You, J.L., and Zhou, S.N. (2002). Endophytic fungi from *Musa acuminata* leaves and roots in South China. World J. Microbiol. Biotechnol. *18,* 169-171.

Carbone, I., and Kohn, L.M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia Carrigy, M.A., ed.*91,* 553-556.

Carroll, G. (1988). Fungal endophytes in stems and leaves - from latent pathogen to mutualistic symbiont. Ecology *69,* 2-9.

Cavagnaro, T.R., Rinaudo, G., Diem, H.G., Auguy, F., Fernandez, D., Bogusz, D., Franche, C., Dommergues, Y., and Huguenin, B. (2001). Quantitative development of Paris-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum* (vol 149, pg 105, 2001). New Phytol. *151,* 551-551.

Chalaturnyk, R.J., Scott, D.J., Baki, Z. (2002). Management of oil sands tailings. Petroleum Science and Technology, Vol. *20 (9 & 10)*, 1025-1046.

Chaverri, P., Castlebury, L.A., Samuels, G.J., and Geiser, D.M. (2003b). Multilocus phylogenetic structure within the *Trichoderma harzianum*. Mol. Phylogenet. Evol. *27,* 302-313.

Chaverri, P., Castlebury, L. A., Overton, B.E., Samuels, G. J. (2003a). *Hypocrea*/*Trichoderma*: species with conidiophore elongations and green conidia. Mycologia. *95*, 1100–1140.

Clay, K., and Holah, J. (1999). Fungal endophyte symbiosis and plant diversity in successional fields. Science *285,* 1742-1744.

Clay, K., Marks, S., and Cheplick, G.P. (1993). Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. Ecology *74,* 1767-1777.

Clay, K., and Schardl, C. (2002). Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat. *160,* S99-S127.

Collado, J.; Platas, G.; Pela'ez, F., (1996). Fungal endophytes in leaves, twigs and bark of Quercusilex from central Spain. Nova Hedwigia *63*, 347–360.

Cox, G., and Tinker, P. B. (1976). Translocation and transfer of nutrients in vesicular arbuscular mycorrhizas the arbuscule and phosphorus transfer a quantitative ultrastructural study. New Phytol. *77*, 371-378.

de Freitas, R., verbal communication (2007)

Dodd, S.L., Crowhurst, R.N., Rodrigo, A.G., Samuels, G.J., Hill, R.A., and Stewart, A. (2000). Examination of *Trichoderma* phylogenies derived from ribosomal DNA sequence data. Mycol. Res. *104,* 23-34.

Douds, D.D., and Schenck, N.C. (1991). Germination and hyphal growth of vam fungi during and after storage in soil at 5 matric potentials. Soil Biology & Biochemistry *23,* 177-183.

Druzhinina, I.S., Kopchinskiy, A.G., Komon, M., Bissett, J., Szakacs, G., and Kubicek, C.P. (2005). An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genetics and Biology *42,* 813-828.

Druzhinina, I., Kubicek, C.P. (2005). Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters. J Zhejiang Univ Sci B. 6(2), 100–112.

Ellis, M. B. & Ellis, J. P. (1985). Microfungi on land plants: An identification handbook. Macmillan Publ. Co.: New York.

Engelhardt, R. and Todirescu, M. (2005). Canada's oil sands: Opportunities and challenges to 2015. An Introduction to Development in Alberta's Oilsand. Calgary: National Energy Board, University of Alberta.

Eom, A.H., Hartnett, D.C., Wilson, G.W.T., and Figge, D.A.H. (1999). The effect of fire, mowing and fertilizer amendment on arbuscular mycorrhizas in tallgrass prairie. Am. Midl. Nat. 142, 55-70.

Ernst M, Mendgen KW, Wirsel SGR. (2003). Endophytic fungal mutualists: Seed-borne *Stagonospora* spp. enhance reed biomass production in axenic microcosms. Molecular Plant-Microbe Interactions 16(7): 580-587.

Eschard, R. and Huc, A.Y. (2008). Habitat of biodegraded heavy oils: Industrial implications. Oil & Gas Science and Technology. Vol. 63 (5), 587-607.

Estaun, M.V. (1990). Effect of sodium-chloride and mannitol on germination and hyphal growth of the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. Agriculture Ecosystems & Environment 29, 123-129.

Franke, M., and Morton, J. (1994). Ontogenic comparisons of arbuscular mycorrhizal fungi scutellospora-heterogama and scutellospora-pellucida-revision of taxonomic character concepts, species descriptions, and phylogenetic hypotheses. Canadian Journal of Botany-Revue Canadienne De Botanique *72,* 122-134.

Freeman, S., and Rodriguez, R.J. (1993). Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science *260,* 75-78.

Gallaud, I. (1905). Etudes sur les mycorrhizes endotrophes. Revue Generale De Botanique. *17*, 5-48, 66-83, 123-136, 223-239, 313-325, 425-433, 479-500.

Garcia Romera, I., Garcia Garrido, J.M., and Ocampo, J.A. (1991). Pectolytic enzymes in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. FEMS Microbiol. Lett. *78,* 343-346.

Garciagarrido, J.M., Garciaromera, I., and Ocampo, J.A. (1992). Cellulase production by the vesicular arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol and Gerd) Gerd and Trappe. New Phytol. *121,* 221-226.

Gardes, M., and Bruns, T.D. (1993). Its primers with enhanced specificity for Basidiomycetes - Application to the identification of mycorrhizae and rusts. Mol. Ecol. *2,* 113-118.

Garg, N., Geetanjali, and Kaur, A. (2006). Arbuscular mycorrhiza: Nutritional aspects. Archives of Agronomy and Soil Science *52,* 593-606.

Gentes, M.L., Waldner, C., Zsuzsanna, P., Smits, J.E.G. (2006). Effects of oil sands tailings compounds and harsh weather on mortality rates, growth and detoxification efforts in nestling tree swallows (*Tachycineta bicolor*). Environmental Pollution. *Vol. 142 (1)*, 24-33.

Girlanda, M., Selosse, M.A., Cafasso, D., Brilli, F., Delfine, S., Fabbian, R., Ghignone, S., Pinelli, P., Segreto, R., Loreto, F., Cozzolino, S., and Perotto, S. (2006). Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal *Russulaceae*. Mol. Ecol. *15,* 491-504.

Goehre, V., and Paszkowski, U. (2006). Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta (Berlin) *223,* 1115-1122.

Harman, G.E. (2000). Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzinum* T-22. Plant Disease *84:4*, 377-393.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species - Opportunistic, avirulent plant symbionts. Nature Reviews Microbiology *2,* 43-56.

Hamel, C. (2004). Impact of arbuscular mycorrhizal fungi on N and P cycling in the root zone. Can. J. Soil Sci. *84*, 383–395.

Hein, F.J. (2006). Heavy oil and oil (Tar) sands in North America: An overview & summary of contributions. Natural resource research. *15*, 66-84.

Hein, F.J. (2000). Historical overview of the Fort McMurray Area and oil sands industry in northeast Alberta. Earth Sciences Report 2000-05. Alberta Geological Survey.

Hendershot, W. H., Lalande, H. and Duquette, M. (1993).Soil reactions and exchangeable acidity. In: Carter, M. ed. Soil sampling and methods of analysis. Lewis Publishers, Boca Raton, Florida, USA. 141–146.

Hepper, C.M., and Warner, A. (1983). Role of organic-matter in growth of a vesicular-arbuscular mycorrhizal fungus in soil. Transactions of the British Mycological Society 81, 155-156.

Hill, N.S., Belesky, D.P., and Stringer, W.C. (1998). Encroachment of endophyte-infected on endophyte-free tall fescue. Annals of Botany *81,* 483-488.

Hijri, I., Sykorova, Z., Oehl, F., Ineichen, K., Mader, P., Wiemken, A. and Redecker, D. (2006). Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Mol. Ecol. *15*, 2277-2289.

Inbar, J., Menendez, A., and Chet, I. (1996). Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. Soil Biology & Biochemistry *28,* 757-763.

Jarstfer, A.G., Farmer-Koppenol, P., Sylvia, D.M., (1998). Tissue magnesium and calcium affect arbuscular mycorhiza development and fungal reproduction, Mycorrhiza *7*, 237–242.

Ju, Y., Sacalis, J.N., and Still, C.C. (1998). Bioactive flavonoids from endophyte-infected blue grass (Poa ampla). J. Agric. Food Chem. *46,* 3785-3788.

Karl, A.C., (1963). Athabasca oil sands. Carrigy, M.A., ed. Athabasca oil sands. Edmonton, Alberta, Canada: Research Council of Alberta.

Kaminskyj, S.G.W. **(**2008). Effective and flexible methods for visualizing and quantifying endorhizal fungi. In Z.A., Akhtar, M.S., Futai, K., eds. Mycorrhizae: Sustainable Agriculture and Forestry Siddiqui. Springer-Verlag, Dordrecht, The Netherlands.

Kapoor, R., Sharma, D., and Bhatnagar, A.K. (2008). Arbuscular mycorrhizae in micropropagation systems and their potential applications. Scientia Horticulturae *116,* 227-239.

Kernaghan, G., Hambling, B., Fung, M., Khasa, D. (2002). *In Vitro* selection of boreal ectomycorrhizal fungi for use in reclamation of saline-alkaline habitats. Restoration Ecology. *Vol. 10 (1)*, 43-51.

Khade, S.W. (2008). Taxonomy of arbuscular mycorrhizal fungi. Mycorrhiza News *20,* 2-7.

Kindermann, J., El-Ayouti, Y., Samuels, G. J. Kubicek, C.P. (1998). Phylogeny of the genus *Trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA clade. Fungal Genet Biol. *24*, 298–309.

Klironomos, J.N., Ursic, M., Rillig, M., and Allen, M.F. (1998). Interspecific differences in the response of arbuscular mycorrhizal fungi to *Artemisia tridentata* grown under elevated atmospheric CO_2. New Phytol. *138,* 599-605.

Kormanik, P.P., Bryan, W.C., and Schultz, R.C. (1980). Procedures and equipment for staining large numbers of plant-root samples for endomycorrhizal assay. Can. J. Microbiol. *26,* 536-538.

Koske, R.E., and Gemma, J.N. (1997). Mycorrhizae and succession in plantings of beachgrass in sand dunes. Am. J. Bot. *84,* 118-130.

Kraus, M., Fusseder, A., and Beck, E. (1987). Development and replenishment of the P-depletion zone around the primary root of maize during the vegetation period. Plant Soil *101,* 247-255.

Krings, M., Taylor, T.N., Hass, H., Kerp, H., Dotzler, N., and Hermsen, E.J. (2007). Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. New Phytol. *174,* 648-657.

Kubiszewski, I., Cutler, J.C., Lawrence, T. (2008). Athabasca, Alberta. In: Encyclopedia of Earth. Cutler, J. Eds. Cleveland (Washington, D.C. Environmental Information Coalition, National Council for Science and the Environment).

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Borner, T., and Kubicek, C.P. (1996). Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc. Natl. Acad. Sci. U. S. A. *93,* 7755-7760.

Kuhn, K.D., (1991). Distribution of vesicular-arbuscular mycorrhizal fungi on a fallow agriculture site. II. Wet habitat. Angew Bot *65*, 187–203.

Kuhn, D.K., Weber, H.C., Dehne, H.W., Gworgwor, N.A. (1991). Distribution of vesicular-arbuscular mycorrhizal fungi on a fallow agriculture site. I. Dry habitat. Angew Bot *65*, 169–185.

Kullnig, C. M., Krupica, T., Woo, S. L., Mach, R. L., Rey, M., Lorito, M. and Kubicek, C. P. (2001). Confusion abounds over identities of *Trichoderma* biocontrol isolates. Mycol. Res. *105*, 770-771.

Kullnig-Gradinger, C. M., Szakacs, G., Kubicek, C. P. (2002). Phylogeny and evolution of the fungal genus *Trichoderma*–a multigene approach. Mycol Res. *106*, 757–767.

Lawrence S. (2008). Oil sands cleanup. National Post. http://energy.probeinternational.org/energy-probe-news/oil-sands-cleanup.

Lehtonen, P., Helander, M., and Saikkonen, K. (2005). Are endophyte-mediated effects on herbivores conditional on soil nutrients? Oecologia *142,* 38-45.

Leuchtmann, A., Schmidt, D., and Bush, L.P. (2000). Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloe*. J. Chem. Ecol. *26,* 1025-1036.

Leung, H.M., Ye, Z.H., and Wong, M.H. (2007). Survival strategies of plants associated with arbuscular mycorrhizal fungi on toxic mine tailings. Chemosphere *66,* 905-915.

Lieckfeldt, E., Kullnig, C.M., Kubicek, C.P., Samuels, G.J., and Borner, T. (2001). *Trichoderma aureoviride*: phylogenetic position and characterization. Mycol. Res. *105,* 313-322.

Lieckfeldt, E., Samuels, G.J., Borner, T., and Gams, W. (1998). *Trichoderma koningii*: neotypification and Hypocrea teleomorph. Canadian Journal of Botany-Revue Canadienne De Botanique *76,* 1507-1522.

Lieckfeldt, E., and Seifert, K.A. (2000). An evaluation of the use of ITS sequences in the taxonomy of the Hypocreales. Stud. Mycol. 35-44.

Lorito, M., Farkas, V., Rebuffat, S., Bodo, B., and Kubicek, C.P. (1996). Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. J. Bacteriol. *178,* 6382-6385.

Lussenhop, J., Treonis, A., Curtis, P.S., Teeri, J.A., Vogel, C.S. (1998). Response of soil biota to elevated atmospheric CO_2 in poplar model systems. *Oecologia 113*, 247-251.

Malinowski, D.P., and Belesky, D.P. (2000). Adaptations of endophyte-infected cool-season grasses to environmental stresses: Mechanisms of drought and mineral stress tolerance. Crop Sci. *40,* 923-940.

Marks, S., Clay, K., and Cheplick, G.P. (1991). Effects of fungal endophytes on interspecific and intraspecific competition in the grasses *Festuca arundinacea* and *Lolium perenne*. J. Appl. Ecol. *28,* 194-204.

Marquez, L.M., Redman, R.S., Rodriguez, R.J., and Roossinck, M.J. (2007). A virus in a fungus in a plant: Three-way symbiosis required for thermal tolerance (vol 315, pg 513, 2007). Science *316,* 201-201.

McGonigle, T.P., and Miller, M.H. (1996). Mycorrhizae, phosphorus absorption, and yield of maize in response to tillage. Soil Sci. Soc. Am. J. *60,* 1856-1861.

Miyasaka, S.C., and Habte, M. (2001). Plant mechanisms and mycorrhizal symbioses to increase phosphorus uptake efficiency. Commun. Soil Sci. Plant Anal. *32,* 1101-1147.

Morton, J.B., and Benny, G.L. (1990). Revised classification of arbuscular mycorrhizal fungi (Zygomycetes) - a new order, Glomales, 2 new suborders, Glomineae and Gigasporineae, and 2 new Families, Acaulosporaceae and Gigasporaceae, with an Emendation of Glomaceae. Mycotaxon *37,* 471-491.

Nagahashi, G., and Dodus, D.,Jr. (1998). Signaling and recognition events prior to colonization of roots by arbuscular mycorrhizal fungi. Phytopathology *88,* S66.

Nagahashi, G., Douds, D.D., and Abney, G.D. (1996). Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza *6,* 403-408.

Naseby, D.C., Pascual, J.A., and Lynch, J.M. (2000). Effect of biocontrol strains of *Trichoderma* on plant growth, pythium ultimum populations, soil microbial communities and soil enzyme activities. J. Appl. Microbiol. *88,* 161-169.

Nogueira, M.A., and Cardoso, E.J.B.N. (2006). Plant growth and phosphorus uptake in mycorrhizal rangpur lime seedlings under different levels of phosphorus. Pesquisa Agropecuaria Brasileira *41,* 93-99.

Nogueira, M.A., and Cardoso, E.J.N. (2007). Phosphorus availability changes the internal and external endomycorrhizal colonization and affects symbiotic effectiveness. Scientia Agricola *64,* 295-300.

Norman, J.R., Atkinson, D., and Hooker, J.E. (1996). Arbuscular mycorrhizal fungal-induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytophthora fragariae*. Plant Soil *185,* 191-198.

O'Donnell, K. (1992). Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium Sambucinum* (*Gibberella Pulicaris*). Curr. Genet. *22,* 213-220.

O'Donnell, K., Cigelnik, E., and Nirenberg, H.I. (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia *90,* 465-493.

O'Donnell, K., Kistler, H.C., Cigelnik, E., and Ploetz, R.C. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. U. S. A. *95,* 2044-2049.

Ormsby, A., Hodson, E., Li, Y., Basinger, J., and Kaminskyj, S. (2007). Quantitation of endorhizal fungi in high Arctic tundra ecosystems through space and time: The value of herbarium archives. Canadian Journal of Botany-Revue Canadienne De Botanique *85,* 599-606.

Paszkowski, U. (2006). A journey through signaling in arbuscular mycorrhizal symbioses. New Phytol. *172,* 35-46.

Peterson, R.L., Massicotte, H.B., and Melville, L.H. (2004). Mycorrhizas: Anatomy and cell biology. Mycorrhizas: Anatomy and Cell Biology *2*, 173.

Petrini, O. (1986). Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema, N.J.and J.Van Den Heuvel Ed.Microbiology of the Phyllosphere. Cambridge University Press: New York 175-187.

Pirozynski, K.A., and Malloch, D.W. (1975). The origin of land plants a matter of mycotrophism. BioSystems *6,* 153-164.

Polishook, J. D., Bills, G. F., Lodge, D. J., (1996).Microfungi from decaying leaves of two rain forest trees in Puerto Rico. J. Ind. Microbiol. *17*, 284–294.

Porter, J.K., Bacon, C.W., and Robbins, J.D. (1979). Ergosine, ergosinine, and chanoclavine-i from *Epichloe typhina*. J. Agric. Food Chem. *27,* 595-598.

Price, M. and Adam, M. (2008). The tar sands. February 21, 2008, Ottawa Citizen Special. Ottawa, Canada.

Rastogi, R., and Sawhney, V.K. (1990). Polyamines and flower development in the male sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum* Mill.) II. Effects of polyamines and their biosynthetic inhibitors on the development of normal and mutant floral buds cultured *in Vitro*. Plant Physiology (Rockville) *93,* 446-452.

Radler, M. (2002). Worldwide report: Worldwide reserves increase as production holds steady. Oil & Gas Journal. *Vol. 100 (52)*, 113.

Read, D.J. (1999). Mycorrhiza – the state of the art. In: Varma A, Hock B, eds. *Mycorrhiza*. Berlin, Germany: Springer-Verlag, 3–34.

Redecker, D. (2000a). Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza *10,* 73-80.

Redecker, D., Hijri, I., and Wiemken, A. (2003). Molecular identification of arbuscular mycorrhizal fungi in roots: Perspectives and problems. Folia Geobotanica *38,* 113-124.

Redecker, D., Kodner, R., and Graham, L.E. (2000b). Glomalean fungi from the Ordovician. Science (Washington D C) *289,* 1920-1921.

Redecker, D., Thierfelder, H., Walker, C., and Werner, D. (1997). Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order glomales. Appl. Environ. Microbiol. *63,* 1756-1761.

Redman, R., and Rodriguez, R. (2007). The population dynamics and symbiotic lifestyle of fungal endophytes in plant hosts is driven by environmental conditions. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology *146,* 220.

Redman, R.S., Dunigan, D.D., and Rodriguez, R.J. (2001). Fungal symbiosis from mutualism to parasitism: Who controls the outcome, host or invader? New Phytol. *151,* 705-716.

Redman, R.S., Ranson, J.C., and Rodriguez, R.J. (1999a). Conversion of the pathogenic fungus *Colletotrichum magna* to a nonpathogenic, endophytic mutualist by gene disruption. Mol. Plant-Microbe Interact. *12,* 969-975.

Redman, R.S., Freeman, S., Clifton, D.R., Morrel, J., Brown, G., and Rodriguez, R.J. (1999b). Biochemical analysis of plant protection afforded by a nonpathogenic endophytic mutant of *Colletotrichum magna*. Plant Physiology (Rockville) *119,* 795-804.

Redman, R.S., Sheehan, K.B., Stout, R.G., Rodriguez, R.J., and Henson, J.M. (2002). Thermotolerance generated by plant. Science (Washington DC) *298,* 1581.

Rice, J.S., Pinkerton, B.W., Stringer, W.C., and Undersander, D.J. (1990). Seed production in tall fescue as affected by fungal endophyte. Crop Sci. *30,* 1303-1305.

Rillig, M.C. (2004b). Arbuscular mycorrhizae, glomalin and soil quality. Canadian Journal of Soil Science 84, 355–363.

Rillig, M.C., Lutgen, E.R., Ramsey, P.W., Klironomos, J.N., Gannon, J.E. (2005). Microbiota accompanying different arbuscular mycorrhizal fungal isolates influence soil aggregation. Pedobiologia 49, 251–259.

Rodriguez, R.J., Henson, J., Van Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y.O., and Redman, R.S. (2008b). Stress tolerance in plants via habitat-adapted symbiosis. ISME Journal *2,* 404-416.

Rodriguez, R.J., Redman, R.S., and Henson, J.M. (2004). The role of fungal symbioses in the adaptation of plants to high stress environments. Mitigation Adapt. Strat. Global Change *9,* 261-272.

Rodriguez, R.J., Redman, R.S., Henson, J.M. (2005). Symbiotic lifestyle expression by fungal endophytes and the adaptation of plants to stress: Unraveling the complexities of intimacy. In: Dighton J., Oudemans P., White J., eds. The Fungal Community: Its organization and role in the ecosystem. Taylor & Francis/CRC Press: Boca Raton, 683–696.

Rodriguez, R., and Redman, R. (2007). More than 400 million years of evolution and plants still can't make it on their own: Plant stress tolerance and habitat expansion via fungal symbiosis. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology *146,* 221.

Rodriguez, R., and Redman, R. (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J. Exp. Bot. *59,* 1109-1114

Rodriguez, R.J., and Redman, R.S. (1997). Fungal life-styles and ecosystem dynamics: Biological aspects of plant pathogens, plant endophytes and saprophytes. Advances in Botanical Research Incorporating Advances in Plant Pathology, Vol *24,* 169-193.

Rodriguez, R.J., White, J.F., Arnold, A.E., Redman, R.S. (2008a). Fungal endophytes: Diversity and functional roles. New Phytol. Tansley review, Vol *182*, 314-330.

Rodriguez-Echeverria, S., Hol, W.H.G., Freitas, H., Eason, W.R., and Cook, R. (2008). Arbuscular mycorrhizal fungi of *Ammophila arenaria* (L.) link: Spore abundance and root colonisation in six locations of the European coast. Eur. J. Soil Biol. *44*, 30-36.

Saikkonen, K., Faeth, S.H., Helander, M., and Sullivan, T.J. (1998). Fungal endophytes: A continuum of interactions with host plants. Annu. Rev. Ecol. Syst. 319-343.

Sambrook, J., Russell, D.W., Sambrook, J., and Russell, D.W. (2001). Molecular cloning: A laboratory manual.

Samuels, G., Dodd, S.L., Gams, W., Castlebury, L.A., and Petrini, O. (2002). *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia *94,* 146-170.

Samuels, G.J., Pardo-Schultheiss, R., Hebbar, K.P., Lumsden, R.D., Bastos, C.N., Costa, J.C., and Bezerra, J.L. (2000). *Trichoderma stromaticum* sp nov., a parasite of the cacao witches broom pathogen. Mycol. Res. *104,* 760-764.

Samuels, G.J., Suarez, C., Solis, K., Holmes, K.A., Thomas, S.E., Ismaiel, A., and Evans, H.C. (2006). *Trichoderma theobromicola* and *T. paucisporum*: two new species isolated from cacao in South America. Mycol. Res. *110,* 381-392.

Santamaria, O., and Diez, J.J. (2005). Fungi in leaves, twigs and stem bark of *Populus tremula* from northern Spain. For. Pathol. *35,* 95-104.

Schardl, C.L., Leuchtmann, A., and Spiering, M.J. (2004). Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology *55,* 315-340.

Schulz, B., Rommert, A.K., Dammann, U., Aust, H.J., and Strack, D. (1999). The endophyte-host interaction: A balanced antagonism? Mycol. Res. *103,* 1275-1283.

Schussler, A., Schwarzott, D., and Walker, C. (2001). A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol. Res. *105,* 1413-1421.

Schutzendubel, A., and Polle, A. (2002). Plant responses to abiotic stresses: Heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. *53,* 1351-1365.

Schwarzott, D., Walker, C., and Schussler, A. (2001). *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is nonmonophyletic. Mol. Phylogenet. Evol. *21,* 190-197.

Shepherd, M., Nguyen, L., Jones, M.E., Nichols, J.D., and Carpenter, F.L. (2007). A method for assessing arbuscular mycorrhizal fungi group distribution in tree roots by intergenic transcribed sequence variation. Plant Soil *290,* 259-268.

Simon, L. (1996). Phylogeny of the Glomales: Deciphering the past to understand the present. New Phytol. *133,* 95-101.

Smith, F.A., and Smith, S.E. (1997). Structural diversity in (vesicular)-arbuscular mycorrhizal symbioses. New Phytol. *137,* 373-388.

Smith, S.E., and Smith, F.A. (1990). Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytol. *114,* 1-38.

Smith, S.E. and Read, D.J. (1997). Mycorrhizal symbiosis. 2^{nd}. Academic Press. London.

Stanton, M. (2004). Origin of the lower cretaceous heavy oils (“Tar Sands”) of Alberta. Search and Discovery Article #10071. Calgary, Alberta.

Strullu-Derrien, C., and Strullu, D.G. (2007). Mycorrhization of fossil and living plants. Comptes Rendus Pale, 483-494.

Tadych, M., and Blaszkowski, J. (2000). Succession of arbuscular mycorrhizal fungi in a deflation hollow of the Slowinski National Park, Poland. Acta Soc. Bot. Pol. *69,* 223-236.

Tawaraya, K., Naito, M., and Wagatsuma, T. (2006). Solubilization of insoluble inorganic phosphate by hyphal exudates of arbuscular mycorrhizal fungi. J. Plant Nutr. *29,* 657-665.

Theodorou, M.E., and Plaxton, W.C. (1993). Induction of pyrophosphate-dependent phosphofructokinase by phosphate starvation in seedlings of *Brassica nigra*. Plant Physiology (Rockville) *102,* 163.

Tian, X.L., Cao, L.X., Tan, H.M., Zeng, Q.G., Jia, Y.Y., Han, W.Q., and Zhou, S.N. (2004). Study on the communities of endophytic fungi and endophytic actinomycetes from rice and their antipathogenic activities *in vitro*. World J. Microbiol. Biotechnol. *20,* 303-309.

Tommerup, I.C. (1984). Effect of soil-water potential on spore germination by vesicular arbuscular mycorrhizal fungi. Transactions of the British Mycological Society *83,* 193-202.

Treseder, K.K. (2004). A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. New Phytol. *164,* 347-355.

Treseder, K.K., and Allen, M.F. (2002). Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: A model and field test. New Phytol. *155,* 507-515.

Treseder, K.K., and Allen, M.F. (2000). Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO_2 and nitrogen deposition. New Phytol. *147,* 189-200.

Tudzynski, B., Sharon, A. (2002). Biosynthesis, biological role and application of fungal phyto-hormones. In: Osiewacz, H.D. ed. The *mycota x.* Industrial applications. Berlin: Springer-Verlag, 183-212.

Ursic, M., Peterson, R.L., and Husband, B. (1997). Relative abundance of mycorrhizal fungi and frequency of root rot on *Pinus strobus* seedlings in a southern Ontario nursery. Canadian Journal of Forest Research *27,* 54-62.

Van Bael, S. A., Maynard, Z., Robbins, N., Bischoff, J., Arnold, A. E., Rojas, E., Mejia L. C., Kyllo D. A., and Herre E. A. (2005). Emerging perspectives on the ecological roles of endophytic fungi in tropical plants. In: Dighton, J., Oudemans, P., and White, J., eds. 3rd ed. The fungal community: its organization and role in the ecosystem. CRC press, Boca Raton, FL. 181–191.

Van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., and Gianinazzi-Pearson, V. (1998). Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. *7,* 879-887.

Vaughn, K.C., and Duke, S.O. (1983). Insitu localization of the sites of paraquat action. Plant Cell and Environment *6,* 13-20.

Vierheilig, H., Coughlan, A.P., Wyss, U., and Piche, Y. (1998). Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl. Environ. Microbiol. *64,* 5004-5007.

Vu, T., Hauschild, R., and Sikora, R.A. (2006). *Fusarium oxysporum* endophytes induced systemic resistance against *Radopholus similis* on banana. Nematology *8,* 847-852.

Walker, C. (1983). Taxonomic concepts in the Endogonaceae - Spore wall characteristics in species descriptions. Mycotaxon 18*,* 443-455.

Walker, C. and Schüßler, A. (2004). Nomenclatural clarifications and new taxa in the glomeromycota. Mycological Research*, 108* , 981-982.

Walker, C., Vestberg, M., Demircik, F., Stockinger, H., Saito, M., Sawaki, H., Nishmura, I., and Schubler, A. (2007). Molecular phylogeny and new taxa in the Archaeosporales (Glomeromycota): *Ambispora fennica gen*. sp nov., Ambisporaceae fam. nov., and emendation of Archaeospora and Archaeosporaceae. Mycological Research. *111,* 137-153.

Wang, W.X., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta *218,* 1-14.

Welz, B. (1999). Atomic absorption spectrometry - pregnant again after 45 years. Spectrochimica Acta Part B-Atomic Spectroscopy *54,* 2081-2094.

Westwood, J.H. (2000). Characterization of the *Orobanche-Arabidopsis* system for studying parasite-host interactions. Weed Science. *48,* 742-748.

Weber RWS, Stenger E, Meffert A, Hahn M. (2004). Brefeldin a production by *Phoma medicaginis* in dead pre-colonized plant tissue: A strategy for habitat conquest? Mycological Research 108: 662-671.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., *Et al*.(Ed.).Pcr Protocols: A Guide to Methods and Applications. Academic Press, Inc.: San Diego, California, USA 315-322.

Wilson, D. (1995). Endophyte - the evolution of a term, and clarification of its use and definition. Oikos *73,* 274-276.

Wubet, T., Weiss, M., Kottke, I., and Oberwinkler, F. (2003). Morphology and molecular diversity of arbuscular mycorrhizal fungi in wild and cultivated yew (*Taxus baccata*). Canadian Journal of Botany *81,* 255-266.

Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M., and Kubicek, C.P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genetics and Biology *26,* 131-140.